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Non-identity of reaction centres for pyrophosphatase and toxic actions of cardiotoxin II: The status of cardiotoxin II as a metalloprotein

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Abstract. Cardiotoxin II of the Indian cobra (*Naja naja*) contains approximately four Mg^{2+} per mol. Complete demetallation of the toxin is achieved by three cycles of treatment with ethylenediamine tetraacetate and gel filtration. Reconstitution of toxin by treatment of the apo-protein with Mg^{2+} restores metal content and inorganic pyrophosphatase activity only to the extent of two atoms/mol and 65%, respectively. Use of Mg (II)-EDTA in the reconstitution experiment yields restoration of half the original enzyme activity. Mg^{2+} is required for the inorganic pyrophosphatase action of the toxin. A definitive statement on the non-essentiality of Mg^{2+} for the lethal toxicity of the toxin is not possible at present, although experimental observations indicate that demetallated toxin is as toxic as the native toxin. Based on this and the differing sensitivities of the enzyme and toxic activities of the toxin to heat, it is suggested that the reaction centres in the toxin for the two activities are different and that the pyrophosphatase activity is not causally connected with the lethal toxicity of the toxin.

Keywords. Cardiotoxin II; pyrophosphatase; magnesium; metalloprotein; reaction centres; cobra.

Introduction

Previous work from this laboratory had demonstrated that cardiotoxin II as isolated by chromatography of cobra (*Naja naja*) venom on CM-Sephadex C-25 columns (Achyuthan *et al.*, 1980) was associated with a nearly constant inorganic pyrophosphatase activity, irrespective of whether chromatography was done in the presence or absence of the detergent Triton X-100 (Achyuthan and Ramachandran, 1980). A subsequent report (Achyuthan and Ramachandran, 1981) had recorded in detail the properties relating to the pyrophosphatase action of cardiotoxin II. It was strange that, unlike many pyrophosphatases, no metal (magnesium) requirement for enzyme activity could be demonstrated as neither added Mg^{2+} nor ethylenediamine tetraacetate exerted any pronounced action. The question whether the pyrophosphatase action has any causal relationship to the cardiotoxicity of the protein also remained to be answered. In this paper we report evidence showing that cardiotoxin II is indeed a Mg^{2+}-containing protein that enzymatic and toxic actions of the protein seem causally unrelated and that cardiotoxin II has no apparent requirement for Mg^{2+} for its toxic action.

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Materials and methods

Cardiotoxin II was isolated as described earlier (Achyuthan *et al.*, 1980). Mg (II)-EDTA complex (Pfeiffer and Simmons, 1943) was prepared according to Brunisholz (1957) in 94.2% yield with a Mg^{2+} content of 94.2% of theoretical and titratable carboxyl content of 1.85 groups/mol. It had a solubility of about 1 μmol/ml at 27°C. Sephadex G-10 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals used were of analytical grade and water for use was double distilled/deionized.

Inorganic pyrophosphatase, protein and lethal toxicity (LD_{50}) were determined by the methods of Heppel (1955) at pH 7.2, Lowry *et al.* (1951) and Litchfield and Wilcoxon (1949), respectively. Magnesium was estimated using an atomic absorption spectrophotometer (Varian Techtron Model 1000), and processing of samples (protein, Tris-(hydroxymethyl)-amino methane (Tris), Mg^{2+}-EDTA and blanks) for analysis was done according to Allan (1971).

Demetallation of cardiotoxin II was done as follows. The protein (20 mg) was dissolved in Tris-HCl buffer (0.4 ml, 0.2 M, pH 7) containing EDTA (0.05 M) and incubated at 4°C for 10 h. It was then applied to a column of Sephadex G-10 (0.9 × 80 cm) and elution done using Tris-HCl buffer not containing EDTA. Fractions of 1 ml were collected. The protein appearing in fractions 10-16 was pooled/lyophilized and analyzed. The protein so recovered was dissolved in 0.5 ml 0.2 M sodium acetate solution (adjusted to pH 7.0) containing 0.05 M EDTA and incubated for 10 h at 4°C. The protein was again recovered after passage through a Sephadex G-10 column using 0.1 M sodium acetate solution (pH 7.0) containing no EDTA for elution. After analyses, the remainder of the protein was subjected to one more cycle of EDTA treatment and gel filtration and the demetallated protein recovered and lyophilized. Remetallation of demetallated cardiotoxin II was done by dissolving the protein (3 mg) in 0.5 ml Tris-HCl buffer containing $MgCl_2$ (50 μmol) and incubating at 37 ± 1°C for 4 h. The solution was then passed through the Sephadex G-10 column and the protein eluted with the Tris-HCl buffer to yield 2.1 mg protein (70%).

The effect of metal ions and metal chelates in restoring the enzymatic activity to the toxin was ascertained by the addition of known concentrations of additives to the pyrophosphatase assay tubes.

Results and discussion

The magnesium content of the native toxin and protein obtained after twice- and thrice-repeated cycles of EDTA treament and gel filtration were 1.47%, 0.22% and 0%, respectively. The magnesium content after remetallation of demetallated protein was 0.69%. Thus native cardiotoxin II, as isolated by ion exchange gel chromatography, contained 4.36% or approximately four atoms of Mg^{2+} per mol based on a molecular weight of 6825 calculated from the known amino acid composition (Srinivasa *et al.*, 1982), where: demetallated protein obtained after two cycles of EDTA treatment and gel filtration contained 0.65 atoms/mol and the fully demetallated product none. Remetallation of fully demetallated cardiotoxin II restored Mg^{2+} content to the extent of only 2.05 atoms per mol. Thus the effectiveness of remetallation is only about 50%.

Naja naja atra (Formosan cobra) venom is reported to have an ash content of 2.16%, of which 6.49% is magnesium (Tu, 1977). Other values reported for venom of other cobras fall in the range 2-100 mg/g venom (Kaye, 1960; Meldrum, 1965; Henriques and Henriques, 1971). Our limited analyses, on two samples of *Naja naja* venom from two different sources (Haffkine Institute and Astik Farm), yielded magnesium contents in the range 4.5-9.2 mg/g venom. Thus, it is interesting to find that cardiotoxin II, the more basic of the two cardiotoxins in venom, which is isolated in weight yields of 15% from crude venom may account for 25-50% of the magnesium present in venom. As remetallation, under conditions used, restores the metal content only to the extent of two atoms/mol, one may infer that half the magnesium in the toxin as isolated, is in a loosely bound form perhaps just carried along with the toxin during ion exchange chromatography and not easily removed from it by desalting on Sephadex G-10 for removal of phosphate buffer salts. Nevertheless, the evidence now presented clearly establishes that cardiotoxin II is a magnesium containing metalloprotein.

The ability to demetallate cardiotoxin II enabled observations on the role of Mg^{2+} in toxicity. Table 1 presents data on LD_{50} values for native cardiotoxin II and demetallated toxin. The latter is only 7% less toxic. On first appearance this would mean that Mg^{2+} is not essential for the display of toxicity. But as the route of administration for LD_{50} measurements in mice is intraperitoneal and deaths occur or are observed over a period of 24 h the possibility is there for remetallation *in vivo* caused by Mg^{2+} in the body pool. Observations on the pyrophosphatase activity of native and demetallated cardiotoxin II under various experimental conditions are

Table 1. Effect of demetallation and of heating (60°C) on the toxicity of cardiotoxin II.

Time (min)	LD_{50} (mg/kg mouse)	Slope function	Potency ratio**
Demetallated Cardiotoxin II: B	2.8 (3.33-2.35)*	1.28	0.93
Cardiotoxin II:			
0 (Control)	2.6 (3.09-2.18)*	1.36	1.00**
40	3.2 (4.16-2.46)*	1.50	0.80
60	3.4 (4.08-2.83)*	1.34	0.77
80	4.0 (4.96-3.23)*	1.47	0.65
100	5.0 (6.65-3.76)*	1.56	0.52

* The values in parentheses represent 19/20 confidence limits

** The potency of native cardiotoxin II is taken as 1.00

given in table 2. The effect of varying concentration of Mg^{2+} on the enzym activity of cardiotoxin II put through only one cycle of demetallation shows tha maximal activity (about 65%) is restored at a Mg^{2+} concentration of 12.5 μmol/ml

Table 2. Effect of metal ions, EDTA and metal chelates on the pyrophosphatase activity of native and demetallated cardiotoxin II.

Sample	Additions	Concentration of additive in assay medium (μmol/ml)	Relative pyrophosphatase activity
Cardiotoxin II-control	None	–	100
Cardiotoxin II	Na_2-EDTA	100	73
Demetallated Cardiotoxin IIA*	None	–	15
Demetallated Cardiotoxin IIB*	None	–	7
Demetallated Cardiotoxin IIC*	None	–	0
Demetallated Cardiotoxin IIB	Mn^{2+}	1-20	0
Demetallated Cardiotoxin IIB	Co^{2+}	1-20	0
Demetallated Cardiotoxin IIB	Mg^{2+}	20	75
Demetallated Cardiotoxin IIB	Mg^{2+}+EDTA	20	
		50	53
Demetallated Cardiotoxin IIB	Mg^{2+} (II)-EDTA	1	44
Demetallated Cardiotoxin IIB	Na_2-EDTA	10	5
Demetallated Cardiotoxin IIB	Mg^{2+} (II)-EDTA	1	
	+Na_2-EDTA	9	17

Protein concentration in all experiments was 0.5 mg/ml

* Demetallated Cardiotoxins II: A, B and C refer to protein recovered after one, two and three cycles, respectively, of treatment with EDTA followed by gel-filtration on Sephadex G-10.

at a protein concentration of 0.5 mg/ml. Mg (II)-EDTA at a concentration of 1 μmol/ml restores 44% of the enzyme activity. Variations in the ratio of Mg (II)-EDTA to the substrate, namely pyrophosphate, from 1 : 1 to 1 : 5 did not affect leve of activity regained. Reconstitution of the metal-enzyme at pH 6.5 or 7.5, and assa of enzyme at pH 7.2 as usual, did not lead to better recovery of activity than tha gained by reconstitution and assay at pH 7.2. A 53% regain of activity is seen wher reconstitution for enzyme assay is effected using 50 μmol/ml of EDTA and 2 μmol/ml Mg^{2+}, conditions in which virtually all Mg^{2+} would exist as Mg (II)-EDTA. That Mg (II)-EDTA can to a large extent be effective in taking on the role o free Mg^{2+} is interesting. This observation explains our earlier finding (Achyutha and Ramachandran, 1981) that the activity of cardiotoxin II as a pyrophosphatas is diminished by only 25% on treatment with EDTA. The experimental difficult observed in not being able to restore the original level of enzyme activity in th reconstitution experiments may mean that either ideal conditions fo reconstitution are yet to be established or that the protein on demetallation has undergone some irreversible change. However, the data amply reveal that the magnesium present in cardiotoxin II, at least about half that originally present, is essential for its pyrophosphatase activity. Neither Co^{2+} nor Mn^{2+} are able to replace Mg^{2+} in this regard.

The data on the effect of heat (60°C) on the pyrophosphatase activity and toxicity of cardiotoxin are presented in table 1 and figure 1. In contrast to toxicity, the enzyme activity is more susceptible to heat. Half of it disappears in less than 1 h and almost all of it in 1.5 h (figure 1). The two activities decay at vastly different rates, with the relative sturdiness of the toxic activity of cardiotoxin II attesting further to the rather well-known heat resistance of snake neurotoxins and cardiotoxins in general. That a single polypeptide, cardiotoxin II, should be invested with two apparently different reaction centres (active sites) need not occasion surprise. One could certainly visualise situations in biologically active proteins where more than one function (active site) is fortuitously present, of which the one conformationally more mobile is more susceptible to heat denaturation. Since samples of toxin in which all enzymatic activity has been destroyed still retain about half the original toxicity, one must conclude that the pyrophosphatase activity is not causally related to the toxic action of cardiotoxin II. That is not to say that pyrophosphatases cannot be toxic *per se*.

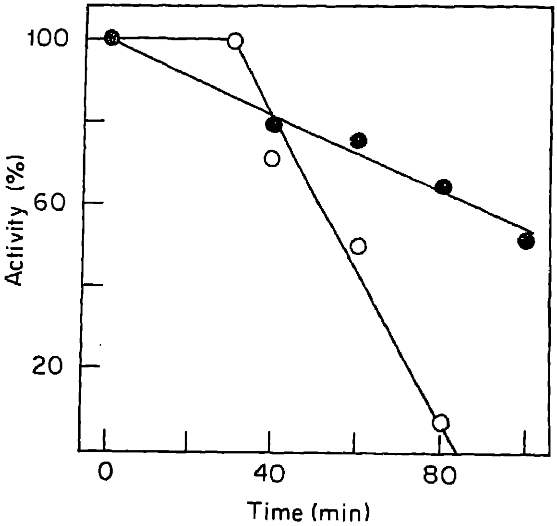

Figure 1. Effect of temperature (60°C) on toxicity and pyrophosphatase activity of Cardiotoxin II. The native protein in water solution was heated in a water bath for different durations. The samples were removed, cooled rapidly and assayed for pyrophosphatase activity (○) and lethal toxicity (LD_{50}) (●).

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Naphthalene oxygenase from *Corynebacterium renale*: Characterisation and mechanism of oxygenation

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Abstract. The formation of *cis*-1,2-dihydroxy-1,2,-dihydronaphthalene from naphthal by naphthalene oxygenase, purified from *Corynebacterium renale* ATCC 15075, demonstrated to involve oxidation of a mol NADH and consumption of one mol oxygen. enzyme contains one g-atom Fe^{2+} and one FAD. Catalase inhibited product formation a H_2O_2 could substitute for NADH in the reaction. Superoxide dismutase inhibited enzy activity when either NADH or H_2O_2 was present; the generation of superoxide anion addition of NADH to the enzyme, in the absence of naphthalene, was detected by the ni blue tetrazolium reduction method. Hydroxyl radical scavengers, ethanol, mannitol a sodium benzoate, inhibited product formation when either NADH or H_2O_2 was prese Electron spin resonance studies, under aerobic conditions, indicated that iron of the enzy underwent valence changes during the course of the reaction.

Keywords. Iron; FAD; hydrogen peroxide; superoxide anion; hydroxyl radical; electr spin resonance.

Introduction

It has been demonstrated that the initial step in the metabolism of aroma hydrocarbons by bacteria is catalysed by iron-containing NAD(P)H-depende dioxygenases and the products formed are *cis*-dihydrodiols (Gibson *et a* 1968 a,b; Axcell and Geary, 1975). Catterall *et al.* (1971) reported th naphthalene dioxygenase from *Pseudomonas* sp. contained tightly bound Fe^{2+} ar was NADPH-dependent. Jeffrey *et al.* (1975) demonstrated the formation of (*cis*- 1,2-dihydroxy-1,2,-dihydronaphthalene from naphthalene using cells ar extracts of *Pseudomonas putida* and established that the enzyme is a dioxygenas Laborde and Gibson (1979) reported that naphthalene dioxygenase fro *Pseudomonas* NCIB 9816 is a multicomponent enzyme system consisting NADH-cytochrome *c* reductase, iron-sulphur protein and a small protein.

At present, the mechanism by which the stereospecific *cis*-1,2-dihydrodiols a formed from many aromatic hydrocarbons by bacterial dioxygenases is not clear understood. The formation of strained dioxetanes (cyclic peroxides) as like intermediates which on reduction could lead to the dihydrodiols has bee postulated by many workers (Kobayashi *et al.*, 1964; Milne *et al.*, 1968; Reiner an

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Hegeman, 1971). Jeffrey, *et al.* (1975) speculated the mechanisms, for naphthalene dioxygenase, involving either the stereospecific introduction of elements of hydrogen peroxide across the C_1-C_2 double bond of naphthalene or the formation of 1,2-dioxetane as an intermediate.

In the present communication, role for NADH, evidence for the generation of superoxide anion and the formation of hydroxyl radical by the mechanism of Haber and Weiss (1934) during the course of naphthalene oxygenase (EC 1:13:99) catalysed reaction is presented. A probable mechanism for the formation of *cis*-1,2-dihydroxyl-1,2,-dihydronapthalene by the enzyme from *C. renale* is proposed.

Materials and methods

The following materials were obtained from the sources specified: Superoxide dismutase (EC 1:15:1:1), catalase (EC 1:11:1:6), peroxidase (EC 1:11:1:7), bovine serum albumin, NADH, FAD, FMN and GSH from Sigma Chemical Co., St. Louis, Missouri, USA; 2,6-dichloroquinone-4-chloroimide, Kieselguhr G, Silica gel G and 1,10-phenanthroline from E. Merck, Darmstadt, Germany; Sephadex G-75 from Pharmacia Fine Chemicals, Uppsala, Sweden; DEAE-cellulose and nitro blue tetrazolium from V.P. Chest Institute, New Delhi. All other chemicals used were of analytical grade.

Enzyme purification

Naphthalene oxygenase was purified to homogeneity from the crude extracts of *Corynebacterium renale* ATCC 15075 as described by Dua and Meera (1981).

Enzyme assay

Naphthalene oxygenase activity was assayed either by measuring oxidation of NADH at 340 nm using Pye-Unicam SP 500 Spectrophotometer or by determining the product formed colorimetrically with 2,6-dichloroquinone-4-chloroimide using-1-naphthol as standard as described by Booth and Boyland (1958).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Determination of iron

Following the method of Brumby and Massey (1967) the iron present in the enzyme (5 mg protein) was extracted into trichloroacetic acid, and then determined colorimetrically with 1,10-phenanthroline using ferrous ammonium sulphate as standard. The essentiality of iron for the enzyme activity was determined by the reconstitution of the apoenzyme, prepared by 1,10-phenanthroline treatment, with Fe^{2+} as described by Nakazawa *et al.* (1969).

Identification and determination of flavin

The flavin moiety of naphthalene oxygenase was liberated by trypsin digestion process as described by Kondo *et al.* (1960) and was used for both its identification and determination. It was identified by TLC on 0.25 mm thick Kieselguhr G coated plate using 5% (w/v) Na_2HPO_4, $12H_2O$ as developing solvent (Fazekas and Kokai, 1971).

The FAD content of the enzyme was determined spectrophotometrically by recording the spectrum of the trypsin digest using Pye-Unicam SP 700 Recording Spectrophotometer and measuring its absorption at 450 nm.

Identification of product

Standard *cis*-1,2-dihydroxy-1,2-dihydronaphthalene was prepared according to the method of Jeffrey *et al.* (1974) and the product of the enzyme reaction was purified and identified by TLC using butan-1-ol/ethanol/water (7 : 1 : 2, by vol) as developing solvent (Meera, 1980).

Electron spin resonance studies

ESR studies were conducted using Varian Model E-12 X-band Spectrometer at 93°K.

Detection of superoxide anion (O_2^{-}) and hydroxyl radicals

The generation of superoxide anion in the enzyme reaction was detected by the method of Beauchamp and Fridovich (1971) which involves inhibition of reduction of nitro blue tetrazolium to formazan by superoxide dismutase. Hydroxyl radical ($\dot{O}H$) formation was detected using scavengers-ethanol, mannitol and sodium benzoate as described by Beauchamp and Fridovich (1970).

Results

Determination of iron

Spectroscopic analysis of the enzyme showed the presence of only iron. Using a molecular weight of 99,000 for the enzyme (as determined by Sephadex G-100 filtration method), the mol ratio of Fe : enzyme was found to be 1 : 1.05. Preincubation of the enzyme with 1,10-phenanthroline and subsequent exhaustive dialysis resulted in 80% loss in activity and the original activity was regained by the addition of Fe^{2+} only (figure 1) which suggested that the enzyme contained Fe^{2+}.

Identification and determination of flavin

The TLC of trypsin digest of naphthalene oxygenase showed the presence of only FAD (table 1). Using a molar extinction coefficient of 11.3×10^3, at 450 nm and molecular weight 99,000 for the enzyme, the mol ratio FAD : enzyme was calculated to be 1 : 1.02 (figure 2).

Effect of catalase

Catalase, although enhanced NADH oxidation, inhibited the product formation by 85%; also, in the absence of naphthalene, catalase caused a small amount of NADH oxidation (table 2).

Effect of hydrogen peroxide

The enzyme catalysed the formation of 1,2-dihydrodiol when NADH was replaced by H_2O_2 in the reaction mixture (table 3). When naphthalene oxygenase was replaced by peroxidase, no product was detected.

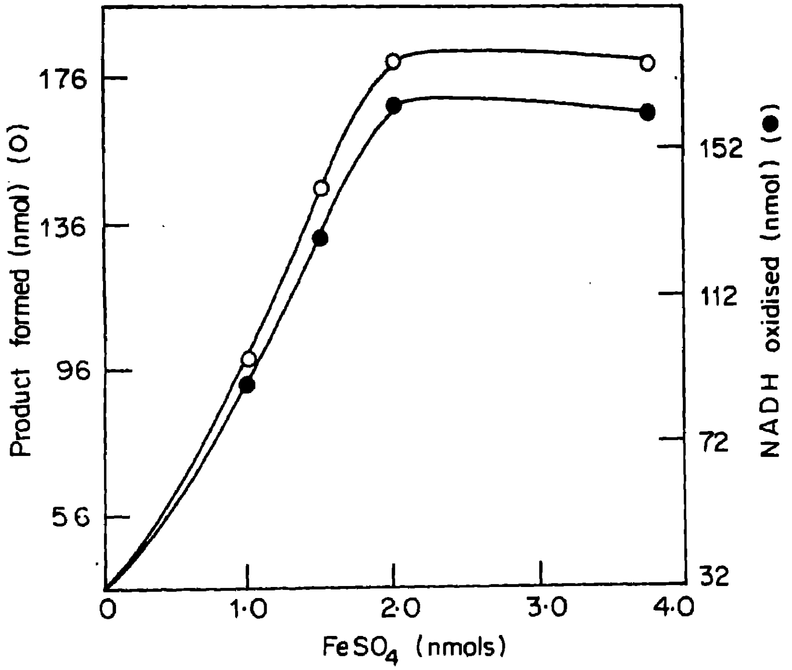


Figure 1. Reconstitution of apoenzyme with iron.

Naphthalene oxygenase (1 mg protein) in 0.05 M phosphate buffer (pH 6.5) was incubated with 2 μmol 1,10-phenanthroline in a total volume of 2.5 ml for 30 min at 40°C and dialysed against 500 ml phosphate buffer (0.05 M; pH 7.0) with three changes for 60 h at 4°C. Aliquots of the dialysed protein were preincubated for 5-10 min at 30°C with different amounts of $FeSO_4$ and assayed for oxygenase activity. The final reaction mixture in each case contained: apoenzyme (0.2 mg protein), 0.8 μmol NADH, 0.2 μmol naphthalene in 0.02 ml 2-methoxyethanol, 0.05 M phosphate buffer (pH 6.5) and different amounts of $FeSO_4$, as indicated, in a total volume of 2.2 ml. The reaction was conducted at 30°C till the oxidation of NADH was complete.

(O) Product formed; (●) NADH oxidised.

Table 1. Identification of flavin.

Compound	R_f
FAD	0.426
FMN	0.298
Riboflavin	0.142
Trypsin digest	0.433
Trypsin digest+FAD	0.433
Trypsin digest+FMN	0.433; 0.30

Purified naphthalene oxygenase (2 mg protein in 2.2 ml 0.05 M phosphate buffer, pH 6.5), after heat denaturation at 80°-100°C for 10 min was digested with 0.3 mg trypsin at pH 8.0 for 5 h at 37°C and treated further as described by Kondo *et al.* (1960). The TLC of the trypsin digest was performed on 0.25 mm thick Kieselguhr G plate using 5% (w/v) $Na_2HPO_4 \cdot 12H_2O$ as developing solvent. The spots were located under u.v. light.

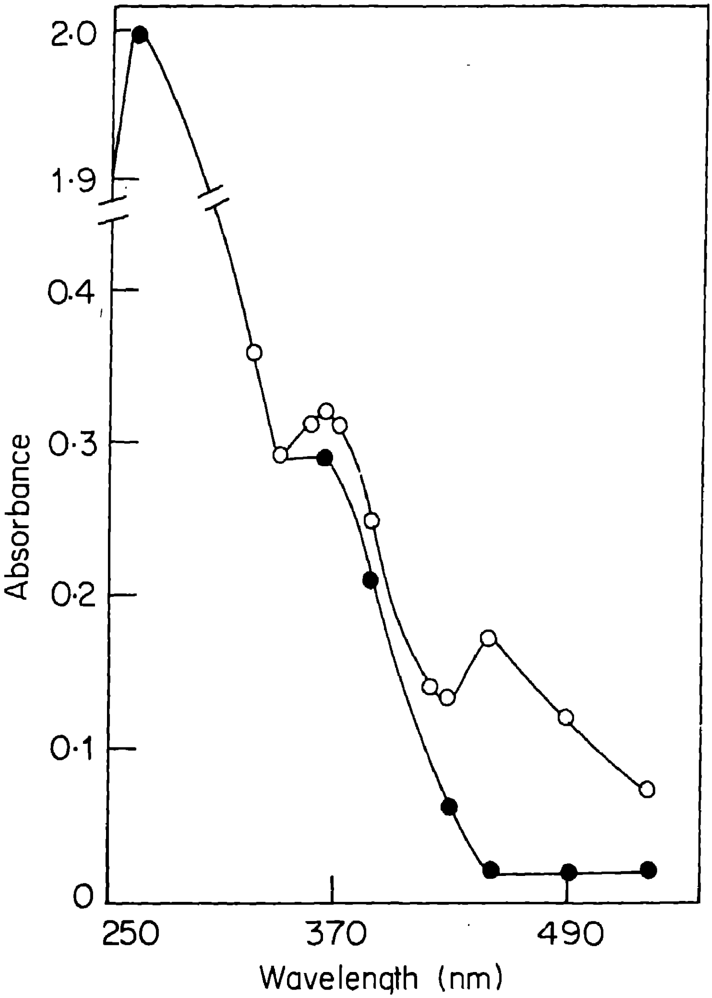


Figure 2. Determination of FAD.

The experimental details were as given in table 1, with the exception that 3 mg oxygenase was used. After digestion, trypsin was destroyed by heating at 80°C for 15 min, the mixture cooled and centrifuged at 2000 *g* for 10 min. The absorption spectrum of the clear supernatant was recorded.

(○) Spectrum of trypsin digest; (●) Spectrum of trypsin digest after addition of 2 mg $Na_2S_2O_4$.

Table 2. Effect of catalase on naphthalene oxygenase catalysed reaction.

Omissions	NADH Oxidised (nmols)	Activity (%)	Product formed (nmols)	Activity (%)
Catalase	190	100	197	100
Nil	226	119	27	14
Naphthalene	49	26	0	0
Naphthalene + Catalase	0	0	0	0

The complete reaction mixture contained: enzyme (0.2 mg protein), 0.8 μmol NADH, 0.2 μmol naphthalene in 0.02 ml 2-methoxyethanol, 100 μg catalase and 0.05 M phosphate buffer (pH 6.5) in a total volume of 2.2 ml. The reaction was conducted for 30 min at 30°C.

Table 3. Effect of substitution of hydrogen peroxide for NADH on product formation.

Addition to control	Amount (μmol)	Product formed (nmol)	Activity (%)
NADH	1	268	100
H_2O_2	1	267	100
	4	282	105.5
	9	307	114
	12	321	120
	18	305	114
	36	250	93.5

Control contained: enzyme (0.2 mg protein), 0.5 μmol naphthalene in 0.05 ml 2-methoxyethanol and 0.05 M phosphate buffer (pH 6.5) in a total volume of 2.2 ml. NADH or H_2O_2 was added as indicated and the reaction was conducted for 15 min at 30°C in a water bath shaker.

Identification of product

The product formed in a reaction mixture containing naphthalene, NADH or H_2O_2, naphthalene oxygenase and phosphate buffer (0.05 M; pH 6.5) had the same R_f (single spot in each case $R_f = 0.87$) as that of standard *cis*-1,2-dihydroxyl-1,2-dihydronaphthalene when subjected to TLC on Silica gel G using butanol/ethanol/water (7 : 1 : 2 by vol) as developing solvent.

Effect of superoxide dismutase

Superoxide dismutase, at 2.73 μM, inhibited the enzyme reaction in terms of both NADH oxidation and product formation by 45%; however, at 6.8 μM, the inhibition was nearly 90% (table 4a). Superoxide dismutase inhibited the reaction even when H_2O_2 was substituted for NADH (table 4b).

Table 4a. Effect of superoxide dismutase on NADH oxidation and product formation.

Additions	NADH oxidised (nmol)	Activity left (%)	Product formed (nmol)	Activity left (%)
Nil	190.0	100.0	197	100.0
Superoxide dismutase (6 nmol)	86.5	45.5	86	43.5
Superoxide dismutase (15 nmol)	18.0	9.5	15	7.5

The reaction mixture contained: enzyme (0.2 mg protein), 0.8 μmol NADH, 0.25 μmol naphthalene in 0.025 ml 2-methoxyethanol and 0.05 M phosphate buffer (pH 6.5) in a total volume of 2.2 ml; superoxide dismutase was added as indicated. The reaction was conducted for 30 min at 30°C.

Table 4b. Effect of superoxide dismutase on naphthalene oxygenase catalysed reaction in the presence of hydrogen peroxide

Additions	Product formed (nmol)	Activity left (%)
Nil	268	100
Superoxide dismutase (15 nmol)	31	14

The reaction mixture contained: enzyme (0.2 mg protein), 1 μmol H_2O_2, 0.5 μmol naphthalene in 0.05 ml 2-methoxyethanol and 0.05 M phosphate buffer (pH 6.5) in a total volume of 2.2 ml; superoxide dismutase was added as indicated. The reaction was conducted for 15 min at 30°C.

Detection of superoxide anion

The enzyme alone did not reduce nitro blue tetrazolium (NBT), but on the addition of NADH, in the absence of naphthalene, reduced NBT which was inhibited by superoxide dismutase by 85% (figure 3). Although, addition of GSH to the enzyme caused some reduction of NBT, no product formation from naphthalene, in the absence of NADH or H_2O_2 was detected. Addition of naphthalene, H_2O_2, mannitol or sodium benzoate did not cause reduction of NBT. These observations indicated the possibility that NADH could be modifying the

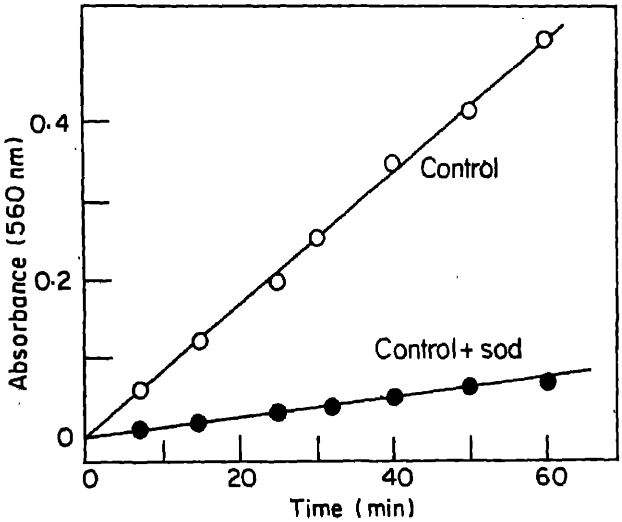


Figure 3. Rate of reduction of NBT by naphthalene oxygenase in the presence of NADH.

The rate of reduction of NBT was measured as change in optical density units at 560 nm. Control contained: enzyme (0.3 mg protein), 1 μmol NADH, 1 μmol NBT and 0.05 M phosphate buffer (pH 6.5) in a total volume of 3.11 ml. Reaction conditions were as given in table 5.

(○) Control; (●) Control plus 6 nmol superoxide dismutase.

naphthalene oxygenase in an allosteric fashion to cause the generation superoxide anion (table 5).

Table 5. Detection of superoxide anion by nitro blue tetrazolium reduction method.

Additions to control	Reduction of NBT (%)	Reduction of NBT in presence of superoxide dismutase (%)
Nil	0	0
NADH	100	13
GSH	50	4
Naphthalene	0	0
H_2O_2	0	0
NADH plus mannitol or benzoate	100	12
Mannitol or benzoate	0	0

Control contained: naphthalene oxygenase (0.3 mg protein), 1 μmol nitro blue tetrazolium (NBT), 0.05 M phosphate buffer (pH 6.5) in a final volume of 3.11 ml. The amounts used for added compounds were: 1 μmol NADH; 4 μmol GSH; 1 μmol naphthalene in 0.1 ml 2-methoxyethanol; 1 μmol H_2O_2; 12 μmol mannitol or benzoate. The reaction mixture was incubated for 60 min at 30°C in a water bath shaker and NBT reduction was measured as optical density units at 560 nm using Spectronic 20 absorptiometer in the presence and absence of 6 nmol superoxide dismutase.

Effect of hydroxyl radical scavengers

Ethanol, mannitol and sodium benzoate inhibited product formation when eith NADH or H_2O_2 was present in the reaction mixture; benzoate had maximu inhibitory effect. Whereas, the inhibition was much greater in presence of H_2 compared to that of NADH for the same concentrations of the scavengers, the was also simultaneous inhibition of NADH oxidation in the NADH syste (table 6). These results suggested the formation of .OH radical in the enzy reaction.

ESR spectral studies

The enzyme alone showed one sharp ESR signal at $g = 2.07$ and on addition NADH, under aerobic conditions, a new signal at $g = 4.3$ appeared which subsequent incubation with naphthalene nearly disappeared while retaining signal at $g = 2.07$ (figure 4). These observations suggested that the iron of enzyme underwent valence changes from Fe^{2+} to Fe^{3+} and back during the cou of the enzyme reaction. ESR studies with initial addition of naphthalene to enzyme could not be conducted as naphthalene was found to separate out freezing.

Effect of ultraviolet irradiation

Irradiation of a mixture containing naphthalene, H_2O_2, naphthalene oxygen and phosphate buffer (0.05 M; pH 6.5) at 240 nm showed that (a) the yield of

Table 6. Effect of .OH radical scavengers on the naphthalene oxygenase catalysed reaction.

		NADH System				H_2O_2 System	
Compound added	Amount (μmol)	NADH Oxidised (nmol)	NADH (Oxidation (per cent inhibition)	Product formed (nmol)	Product formation (per cent inhibition)	Product formed (nmol)	Product formation (per cent inhibition)
–	–	259	0	265	0	265	0
Sodium benzoate	1.0	200	23.0	183	31.0	85	68
	2.0	172	33.5	150	43.5	45	83
	4.0	147	43.0	113	57.5	0	100
	8.0	114	56.0	75	83.0	–	–
Mannitol	1.0	214	17.5	197	25.5	186	30
	2.0	193	25.5	168	36.5	122	54
	4.0	172	33.5	134	49.5	32	88
	8.0	155	40.0	115	56.5	–	–
Ethanol	2×10^2	245	5.5	250	5.5	240	9.5
	10.3×10^2	210	19.0	200	24.5	160	40
	41.5×10^2	180	30.5	160	40.0	80	70

The reaction mixture contained: enzyme (0.2 mg protein), 0.5 μmol naphthalene in 0.05 ml 2-methoxyethanol, 1 μmol either NADH or H_2O_2 and 0.05 M phosphate buffer (pH 6.5) in a total volume of 2.2 ml. The scavengers were added as indicated. The reaction was conducted in each case for 15 min at 30°C.

dihydrodiol was not affected, (b) but when superoxide dismutase was included, some unidentified compounds (which gave phenolic reaction on acid treatment and whose overall yield as 1-naphthol was only 50%) were formed and (c) absorbance at 240 nm in presence and absence of superoxide dismutase also simultaneously decreased. In contrast, in the absence of oxygenase, no change in absorbance at 240 nm was observed, whether superoxide dismutase was present or absent, although some unidentified compounds whose qualitative tests and yield were similar to that of oxygenase system in presence of superoxide dismutase were formed; besides, mannitol and benzoate completely inhibited the formation of these compounds (table 7).

Discussion

Dua and Meera (1981) reported that purified naphthalene oxygenase from *Corynebacterium renale* oxidised a mol NADH for the incorporation of one mol oxygen into naphthalene and that the product of the reaction was *cis*-1,2-dihydroxy-1,2,-dihydronaphthalene. The enzyme was found to contain one g-atom Fe^{2+} and one FAD as determined by colorimetric and absorptiometric methods respectively. The ESR signal at $g = 2.07$, reconstitution of apoenzyme with Fe^{2+} and lack of absorption in the Soret region by the enzyme alone or after its treatment with CO for 5 min in dark indicated the presence of non-heme Fe^{2+}.

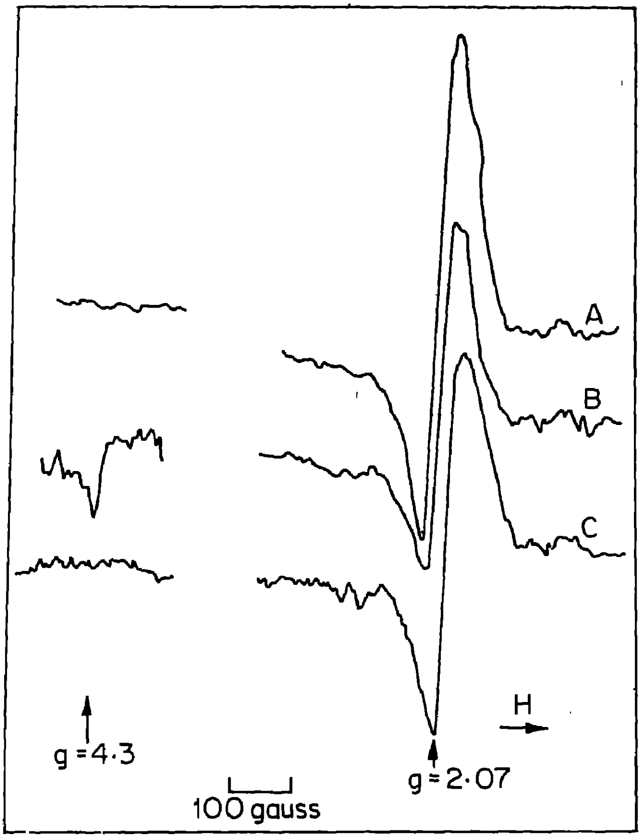


Figure 4. ESR spectrum of naphthalene oxygenase, in presence of air, under different conditions at 93°K; modulation amplitude 5 G.

Enzyme (1 mg protein) dissolved in 0.05 M phosphate buffer (pH 6.5) was frozen and the spectrum recorded. To this enzyme soln., 2 µmol NADH was added, incubated for 20 min at 30°C and the spectrum recorded. Finally, to the NADH preincubated enzyme, 2 µmol naphthalene was added, incubated again for 20 min at 30°C and the ESR spectrum recorded.

(A) Enzyme alone; (B) Enzyme+NADH; (C) Enzyme+NADH+napthalene.

Experiments with catalase, which inhibited product formation significantly but enhanced NADH oxidation suggested that the binding of naphthalene to the enzyme initiated the generation of H_2O_2; subsequent studies confirmed that H_2O_2 itself could substitute for NADH in the enzyme reaction. The enzymatic reaction in presence of either NADH or H_2O_2 was inhibited by superoxide dismutase; although, high amount of superoxide dismutase was required to cause significant inhibition, analysis of the reaction showed that the addition of NADH alone to the enzyme caused the generation of O_2^- as tested by the NBT reduction method. The results suggested that (a) NADH binds to the enzyme initially so as to expose its Fe^{2+} to O_2 and (b) the second function of NADH is to produce H_2O_2 by oxidation *via* FAD of the enzyme only when naphthalene was present. The hydroxyl radical scavengers inhibited product formation when either NADH or H_2O_2 was present; however, there was also simultaneous inhibition of NADH oxidation. At present,

Table 7. Effect of U.V. irradiation on system containing naphthalene and hydrogen peroxide in the presence and absence of naphthalene oxygenase.

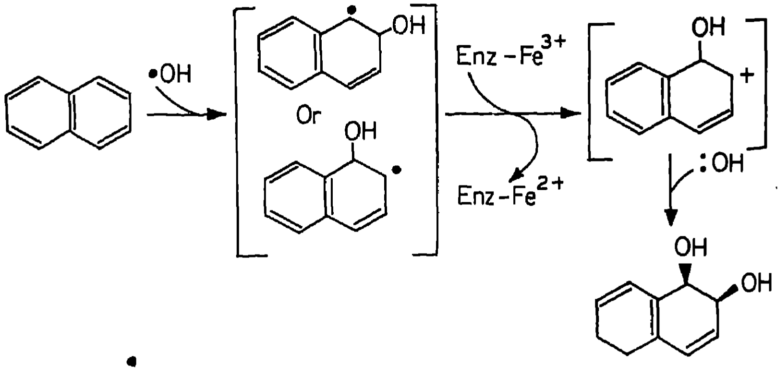
Additions to control	ΔA_{240}	Products as 1-naphthol (nmol)
Enzyme	0.17	495
Enzyme plus superoxide dismutase	0.17	250
Nil	0	253
Superoxide dismutase	0	246
H_2O_2	0	250
Mannitol	0	0
Sodium benzoate	0	0

Control contained: 0.5 μmol naphthalene in 0.05 ml 2-methoxyethanol, 1 μmol H_2O_2 and 0.05 M phosphate buffer (pH 6.5) in a total volume of 2.32 ml. The amounts used for added compounds were: 0.2 mg naphthalene oxygenase; 15 nmol superoxide dismutase; 4 μmol H_2O_2; 4 μmol mannitol or sodium benzoate. The reaction was terminated in each case by addition 0.23ml conc.HCl after irradiating for 30 min at 30°C at 240 nm in a 1 cm cuvette. The products were determined as 1-naphthol after heating for 15 min at 100°C by the method of Booth and Boyland (1958).

we do not know the reason for this unexpected phenomenon but it is likely that the rate of generation of O_2^- was also indirectly affected as the scavengers are not known to react with either O_2^- or H_2O_2 (Fridovich, 1976). The appearance of a new ESR signal at $g = 4.3$, under aerobic conditions, on the addition of NADH to the enzyme, and its near disappearance on subsequent incubation with naphthalene suggested that during the course of the reaction the enz-Fe^{2+} underwent valence changes to Fe^{3+} and back (Meera and Rao, 1979). Also, when NADH was replaced by H_2O_2, there is evidence for the involvement of O_2^- in the enzyme reaction; *viz.* inhibition by superoxide dismutase and decrease in absorbance at 240 nm (ε_{240} for $O_2^- = 1980\ M^{-1}cm^{-1}$; Behar *et al.*, 1970) during the course of the reaction in presence or absence of superoxide dismutase; here, the results suggested that O_2^- generation took place only on the addition of naphthalene to the mixture previously containing H_2O_2 and enzyme. The hydroxyl radical scavengers inhibited this system more than the NADH system. The formation of unidentified compounds on irradiation of the mixture containing naphthalene in 2-methoxyethanol, H_2O_2 and phosphate buffer at 240 nm is likely to be due to .OH radicals, generated by the dismutation of H_2O_2 (Hochanadel, 1962), reacting with naphthalene. The above results suggested (a) operation of regulatory mechanisms in the production of H_2O_2 and O_2^- by the enzyme, (b) that O_2^- is generated by the reaction, Fe^{2+} —— O_2 —— Fe^{3+} —— O_2^- and (c) that .OH radical is produced by the Haber-Weiss (1934) mechanism. We propose the following sequence which involves incorporation of .OH radical into naphthalene in the formation of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene by naphthalene oxygenase from *C. renale.*

$$\text{Enz-Fe}^{2+} - O_2 \longleftrightarrow \text{Enz-Fe}^{3+} - O_2^{-}$$

$$\text{Enz-Fe}^{3+} - O_2^{-} + H_2O_2 \longrightarrow \text{Enz-Fe}^{3+} + \cdot OH + :OH^{-} + O_2$$



The mechanism proposed is consistent with the stoichiometry of the reaction. The slow rate of Haber-Weiss reaction (k = 0.1 to 10 $M^{-1}s^{-1}$) and the subsequent fast addition of O˙H radical to C_1-C_2 double bond of naphthalene (k = (9.5 ± 1.5) × 10^9 $M^{-1}s^{-1}$; Michael and Hart, 1970) ensure that hydroxyl radical does not accumulate in the system. Based on the presumption that naphthalene oxygenase from *C. renale* is a dioxygenase, we postulate that both the hydroxyl groups of *cis*-1,2-dihydrodiol arise from O_2^-. Experiments confirming the validity of the above scheme using $H_2{}^{18}O_2$, $H_2{}^{18}O$ and ${}^{18}O_2$ are in progress. The hydroxyl radical has been implicated in other systems: hydroxylation of *p*-cresol and *p*-nitrophenol (Goscin and Fridovich, 1972) production of ethylene from methional (Beauchamp and Fridovich, 1970); peroxidation of lysosomal membranes (Fong *et al.*, 1973) and deploymerization of hyaluronic acid (McCord, 1974).

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Enzyme inhibitors from plants. Isolation and characterization of a protease inhibitor from arrow root *(Maranta arundinaceae)* tuber

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Abstract. A protease inhibitor from arrow root *(Maranta arundinaceae)* tuber has been isolated in a homogeneous form. The inhibitor has a M_r of 11,000-12,000; it inhibited bovine trypsin, bovine enterokinase, bovine α-chymotrypsin and the proteolytic activity of human and bovine pancreatic preparations. The inhibitor is resistant to pepsin, and elastase. It could withstand heat treatment at 100°C for 60 min and exposure to a wide range of pH (1.0-12.5) for 72 h at 4°C without loss of activity. Arginyl groups are essential for the action of the inhibitor. Preincubation of the inhibitor at pH 3.7 with trypsin or chymotrypsin caused nearly a two-fold increase in inhibitor potency.

Keywords. Arrow root tuber; protease inhibitor; isolation; properties.

Introduction

Protease inhibitors from plant tubers like potato (Ryan, 1973; Bryant *et al.*, 1976; Pearce *et al.*, 1982; Richardson, 1977; Melville and Ryan, 1972), sweet potato (Sugiura *et al.*, 1973), *Alocasia macrorhiza* (Sumathi and Pattabiraman, 1977) and *Colocasia antiquorum* (Sumathi and Pattabiraman, 1979) have been studied extensively. Sumathi (1979) screened several plant tubers and bulbs for antiprotease activity and found that arrow root tuber comes under the group of plant materials with high antiprotease activity. Recently, Bhat *et al.* (1981) reported enterokinase inhibitory activity in this tuber. In view of limited information available on enterokinase inhibitory activity of plant protease inhibitors, it was considered worthwhile to study the inhibitory factors in arrow root tubers in detail. In this communication we report the isolation and properties of a low molecular weight inhibitor from this tuber which is capable of inhibiting trypsin, enterokinase and α-chymotrypsin.

Abbreviations used: BAEE, α-N-benzoyl L-arginine ethyl ester; BAPNA, α-N-benzoyl DL-arginine *p*-nitro anilide; TNBS, trinitro benzene sulphonate; OMI, O-methyl isourea. CHD, cyclohexane dione.

Materials and methods

Arrow root tubers were procured from local sources. Bovine trypsin (salt-free, twice crystallized), bovine α-chymotrypsin (salt free, thrice crystallized) and porcine elastase (twice crystallized) were the products of Worthington Biochemical Corporation, USA. Peroxidase, cytochrome c, myoglobin, α-N-benzoyl L-arginine ethyl ester (BAEE). α-N-benzoyl DL-arginine *p*-nitroanilide (BAPNA), sodium trinitrobenzene sulphonate (TNBS), O-methyl isourea (OMI) and Dalton mark VI were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Porcine pepsin (thrice crystallized) was obtained from Calbiochem, USA. Affigel-10 was the product of Bio-Rad Laboratories, USA. 1,2-Cyclohexanedione (CHD) was obtained from Aldrich Chemicals, USA. Subtilisin BPN was purchased from Nagase Company, Osaka, Japan.

Bovine enterokinase was purified up to the ammonium sulphate stage (Liepnicks and Light, 1979). Bovine pancreas was collected from local slaughter house. Human pancreas was collected during autopsy at the Kasturba Medical College Hospital, Manipal. The tissues were kept in frozen condition until they were processed. Acetone powders of human and bovine pancreas were prepared and used as sources of proenzymes. The preparations were activated using bovine enterokinase (Chandrasekher and Pattabiraman, 1982).

Trypsin-Affigel was prepared as follows. Bovine trypsin (350 mg) in 50 ml of 0.1 M phosphate buffer pH 7.0 was added to 50 ml (wet volume) of Affigel-10 at 4°C and gently stirred for 6 h. After standing overnight at 4°C, the suspension was filtered and washed extensively with 1.0 M ethanolamine-HCl in 0.1 M phosphate buffer pH 7.0 at a flow rate of 50 ml h^{-1} followed by the buffer containing 1.0 M NaCl till there was no absorbance at 260 nm. The immobilized enzyme preparation was stored at 4°C in 50 mM HCl until use.

Caseinolytic assay of the proteolytic enzymes were performed (Sumathi and Pattabiraman, 1975). Ten μg of bovine trypsin, 12.5 μg of bovine α-chymotrypsin, 0.2 mg protein of activated human pancreatic preparation, 0.11 mg protein of activated bovine pancreatic preparation, 12.5 μg of elastase and 20 μg of subtilisin BPN were used in the assay systems to get comparable activities in the linear range. One unit of caseinolytic activity is defined as the amount of enzyme that liberated one mg of trichloroacetic acid-soluble peptides.

Amidolytic activity of trypsin was measured, using BAPNA as substrate according to the method of Erlanger *et al.* (1961). Esterase activity of bovine enterokinase was determined using BAEE as substrate (Bhat *et al.* 1981). Protein content was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

The purified inhibitor was analyzed for carbohydrate content by the method of Dubois *et al.* (1956) using galactose as standard.

Active site titration of bovine trypsin was performed according to Sumathi, (1979). The trypsin preparation was 65% active.

The inhibitor activities were determined by including suitable amounts of inhibitor in the assay system. One inhibitor unit is defined as the amount of inhibitor required to suppress the enzyme activity by one unit. Controls without inhibitor were run simultaneously. Unless mentioned otherwise, the inhibitory activities discussed are those obtained by the caseinolytic method.

Isolation of the major inhibitor

Arrow root tuber was homogenized with 3 volumes (wt/vol) of 0.02 M phosphate buffer pH, 7.6 and the mixture was stirred in the cold (5°C) for 30 min and set aside for 90 min. The suspension was filtered under suction. To the clear filtrate, five volumes of cold (−5°C) acetone was added with stirring. After 20 min stirring, the precipitate was collected by suction filtration, washed with cold acetone and finally with diethyl ether. The precipitate was air dried and stored at 4°C under desiccation. One g of acetone powder (obtained from 30 g of tubers) was suspended in 70 ml of 0.02 M phosphate buffer, pH 7.6. This was designated as the crude extract.

The crude extract was subjected to heat treatment at 90°C for 5 min, cooled to room temperature and centrifuged at 10,000 *g* for 20 min. The supernatant was designated as the heat treated fraction.

To this fraction (vol. 60 ml) solid ammonium sulphate (23.4 g) was added to 60% saturation at 4°C and the suspension was left overnight. The precipitate was collected by centrifugation at 10,000 *g* for 20 min, dissolved in 0.02 M phosphate buffer, pH 7.6, dialyzed against 100 volumes of 2×10^{-3} M phosphate buffer, pH 7.6 for 16 h and centrifuged at 10,000 *g* for 20 min. The clear supernatant (8.0 ml) was designated as the ammonium sulphate fraction. The next three stages of purification were carried out in batches.

DEAE-cellulose chromatography: Four ml of the ammonium sulphate fraction was loaded onto a column of DEAE-cellulose (1.6 × 12.4 cm, bed vol. 25 ml) equilibrated with 2×10^{-3} M phosphate buffer, pH 7.6 at room temperature. The column was washed with 140 ml of the equilibration buffer at a flow rate of 20 ml h^{-1} and the washings containing the inactive proteins were discarded. The column was then eluted with a linear gradient system of NaCl formed between 95 ml of 0.02 M phosphate buffer pH 7.6 in the mixing chamber and 95 ml of the buffer containing 0.8 M NaCl in the reservoir. Ten ml fractions were collected at a flow rate of 20 ml h^{-1}. The active fractions (tube numbers 5 and 6, figure 1) were pooled and designated as DEAE-cellulose fraction.

Affinity chromatography: The above fraction (20 ml) was allowed to flow through a column of trypsin-Affigel (1.6 × 12.4 cm, bed vol. 25.0 ml) equilibrated with 0.02 M phosphate buffer, pH 7.6 containing 0.3 M NaCl at 4°C. The column was washed with 70 ml of this solution at a flow rate of 10.0 ml h^{-1} and the washings were discarded. The column was eluted with 50 mM HCl and ten ml fractions were collected. The active fractions (tube numbers 10 and 11, figure 2) were pooled and designated as trypsin-Affigel fraction.

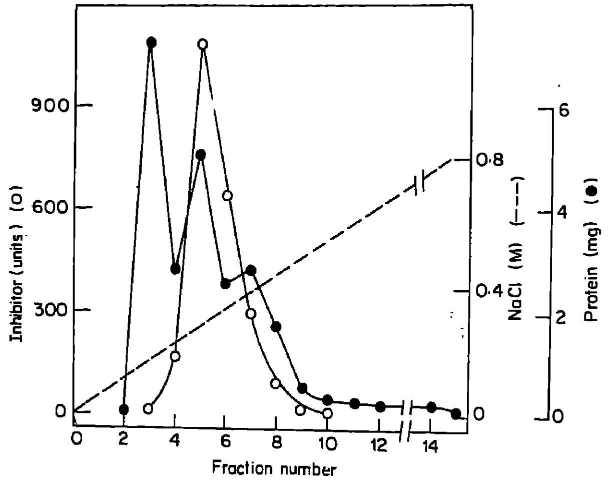

Figure 1. Fractionation of ammonium sulphate fraction on DEAE-cellulose. (Details are given under materials and methods).

(●) Protein content; (O) Antitryptic activity.

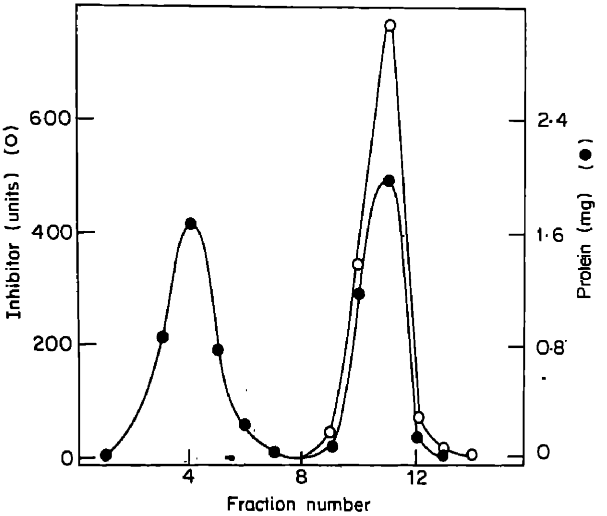

Figure 2. Affinity chromatography of DEAE-cellulose fraction on trypsin-Affigel 10. (Experimental details are given under materials and methods).

(●) Protein content; (O) Antitryptic activity.

Chromatography on Sephacryl S-200: The above fraction was dialysed against 0.04 M phosphate buffer, pH 7.6 at 4°C and concentrated to 4.0 ml by lyophilization. To this solution 70 mg of NaCl was added to give a final concentration of 0.3 M NaCl. This solution was applied to a column of Sephacryl S-200 (1.6 × 62.0 cm, bed vol. 120.0 ml) equilibrated with 0.2 M phosphate buffer, pH 7.6 containing 0.3 M NaCl. The column was eluted at room temperature with the same solution at a flow rate of 8 ml h^{-1} and 2.0 ml fractions were collected. The major active fractions (tube numbers 67-71, figure 3) were pooled and dialyzed against 100 volumes of distilled water at 4°C. This was designated as the Sephacryl fraction and was used for further studies.

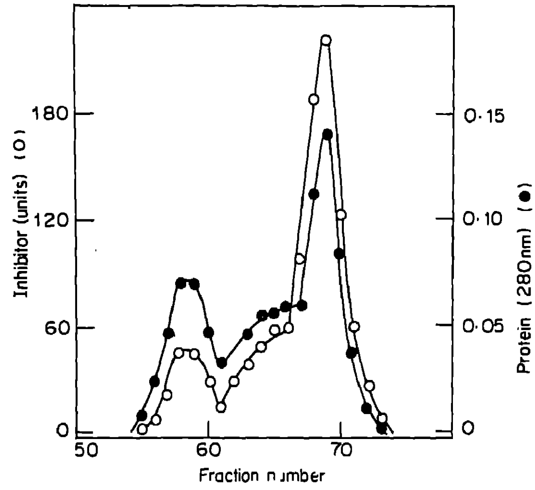

Figure 3. Gel chromatography of trypsin-Affigel 10 fraction on Sephacryl S-200. (Experimental details are given under materials and methods). (●) Absorbance at 280 nm; (O) Trypsin inhibitory activity.

Electrophoresis: Cellulose acetate membrane electrophoresis of the purified inhibitor was performed at pH 8.6 (0.05 M barbitone buffer) and at pH 5.0 (0.5 M acetate buffer) for 75 min at 200 V. The strips were stained with 0.1% Coomassie Brilliant Blue R-250 in 7% acetic acid. Polyacrylamide gel electrophoresis was performed according to the method of Davis *et al.* (1964) at pH 8.3 using 7.0% polyacrylamide. The electrophoresis was performed for 60 min at 3 mA current per tube. Coomassie Brilliant Blue R-250 (0.1% in methanol-acetic acid-water 5:1:5 vol/vol) was used to stain the protein. SDS-Polyacrylamide gel electrophoresis was performed at pH 7.2 according to the method of Weber *et al.* (1972) using 10.0% polyacrylamide. The inhibitor was treated with 1% SDS, 1% mercaptoethanol and 36% urea in 0.01 M phosphate buffer, pH 7.2 at 37°C for 2 h. The electrophoresis was performed for 5 h at 7 mA current per tube. Staining was done as described above.

Molecular weight determination: This was determined by SDS-polyacrylamide gel electrophoresis using Dalton mark VI for standardization. Molecular weight was also determined by gel permeation chromatography on Sephadex G-100 column (0.9×56.5 cm, bed volume 36 ml). The column was equilibrated with 0.02 M phosphate buffer pH 7.6 containing 0.1 *M* NaCl and the same system was used as eluant. The purified inhibitor (150 μg protein) in a volume of 1.0 ml was applied to the column and eluted at a flow rate of 8.0 ml h^{-1} at room temperature. One ml fractions were collected and assayed for protein and antitryptic activity. Cytochrome c, myoglobin, peroxidase, bovine serum albumin and bovine trypsin were used as marker proteins.

Effect of pH on the stability of the inhibitor: The inhibitor (2.9 μg protein) in 0.1 ml water was incubated with 0.1 ml of different solutions or buffers for 72 h at 4°C (pH 1.0) 0.1 M HCl; (pH 2.0) 0.1 M HCl/KCl; (pH 4.5) 0.1 M acetate; (pH 6.0 and 7.0) 0.1 M sodium phosphate; (pH 8.0) 0.1 M Tris-HCl; (pH 9.0) 0.1 M borate; (pH 10.0) 0.1 *M* sodium bicarbonate and (pH 12.5) 0.2 M NaOH. After this treatment, 0.4 ml of 0.2 M phosphate buffer, pH 7.6 was added and aliquots were assayed for antitryptic activity.

Effect of temperature on the stability of the inhibition: The inhibitor (1.45 μg protein) in 20 μl of water was subjected to heat treatment at 10°C for different time intervals, cooled and assayed for residual inhibitor activity against bovine trypsin and α-chymotrypsin. The inhibitor was also subjected to pressure cooking (15 lb/in^2) for 20 min.

Chemical modification: For amino group modification (Haynes *et al.*,1967),14.5 μg protein of the inhibitor was treated with 200 μg of TNBS at pH 7.6 (0.18 mM phosphate) in a volume of 2.0 ml at room temperature for 24 h. The solution was dialysed against one litre of water for 8 h at 4°C and aliquots of the dialysed solution were used for the determination of antitryptic and antichymotryptic activities. OMI also was employed to study the role of amino groups (Plapp *et al.*, 1971). The inhibitor (7.3 μg protein) was allowed to react with 1.0 mg of OMI in a volume of 1.0 ml containing 8.0 μmol of sodium hydroxide (final pH 11.5) for 24 h room temperature. The solution was dialysed and processed as described above.

Guanidino groups were modified by treatment with ninhydrin(Chaplin,1976) and with CHD (Abe *et al.*, 1978). The inhibitor (29.2 μg protein) was treated with 4.0 mg of ninhydrin at 37°C in a volume of 4.0 ml containing 640 μmol of Na_2HPO_4 (final pH 9.2). Aliquots were withdrawn after definite time intervals and dialyzed against 0.1 M acetate buffer pH 4.0 for 5 h at 4°C. The dialyzed solutions were assayed for residual antitryptic and antichymotryptic activities. For CHD treatment, 36 μg of the inhibitor was incubated with either 500 μg 2.0 mg of the reagent in a volume of 5.0 ml containing 900 μmol of borate buffer pH 9.0 at 37°C for definite periods of time. Aliquots were dialysed against one litre of distilled water for 8 h at 4°C and assayed for residual inhibitory activities. The inhibitor was also subjected to heat treatment at 100°C for 10 min in an experiment prior to treatment with CHD. Controls without the chemical modifiers were run in all cases.

Effect of protease on the inhibitor: The inhibitor (2.9 μg protein) was incubated with 3.0 μg of pepsin at 37°C for 6 h at pH 2.0 (0.1 M HCl/KCl buffer) in a volume of 0.25 ml. To this solution, 0.4 ml of 0.2 M phosphate buffer pH 7.6 was added and the solution was directly used for the determination of residual antitryptic activity. For studying the effect of elastase, 5.8 μg of the inhibitor was treated with 12.5 μg of elastase for one h at 37°C in a volume of 0.4 ml in the presence of 80 μmol of phosphate buffer pH 7.6. Aliquots were directly used for antitryptic activity measurement using BAPNA as substrate. Controls without the enzymes were run simultaneously.

Preincubation studies with trypsin and α-chymotrypsin: The inhibitor (2.1 μg protein) was incubated with 10 μg of bovine trypsin for definite time intervals either at 4°C, 25°C, or at 37°C in the presence of 4.0 μmol of acetate buffer pH 3.7 in a volume of 0.5 ml. The pH was adjusted to 7.6 by the addition of 0.5 ml of 0.2 M phosphate buffer and assayed for antitryptic activity by the addition of 1.0 ml of 2% casein solution. Similarly, preincubation was done with 12.5 μg of bovine α-chymotrypsin and the antichymotryptic activity was assayed. Preincubation studies were also done at pH 7.6 using 0.2 M phosphate buffer. Controls without the inhibitor were run simultaneously.

Studies on complex formation: The inhibitor (150 μg protein) was incubated with 250 μg of bovine trypsin for one h at 37°C in the presence of 10 μmol phosphate buffer pH 7.6 and 50 μmol of NaCl in a volume of 0.5 ml. The solution was subjected to gel chromatography on Sephadex G-100 (see molecular weight determination) and the fractions were analyzed for protein, antitryptic and tryptic activites. Trypsin and the inhibitor were also subjected separately to gel chromatography.

Results

A crude extract of arrow root tuber was found to inhibit both bovine trypsin and α-chymotrypsin. While the two activities could not be separated, the inhibitory activity in the tuber could be resolved into two fractions during gel filtration on Sephacryl S-200. Both the fractions showed anti-tryptic and antichymotryptic activities. The low molecular weight, major inhibitor (figure 3) accounted for about 80% of the recovered antitryptic activity.

The major inhibitor was purified about 40 fold and the data on purification are summarized in table 1. The inhibitor was homogeneous by several criteria. On cellulose acetate electrophoresis at pH 9·8 and at pH 5.0, it moved as a single protein band, 26.0 mm and 16.0 mm respectively, towards the anode (data not shown). During SDS-polyacrylamide gel electrophoresis at pH 7.2 and during gel electrophoresis in the absence of SDS at pH 8.3 the inhibitor moved as a single band (figure 4). During gel permeation chromatography on Sephadex G-100, the activity and protein levels corresponded in the individual fractions (figure 5).

The molecular weight of the inhibitor was found to be 12,000 based on SDS-polyacrylamide gel electrophoretic data (figure 6a) and the value of 11,000 based on gel chromatography on Sephadex G-100 agreed fairly well with this value (figure 6b).

Table 1. Purification of the major protease inhibitor from arrow root tuber.

Steps	Total protein (mg)	Inhibitor (units)	Specific activity	Yield (%)
Crude Extract	581	7550	13.0	100.0
Heat treatment	66	5445	82.5	72.0
Ammonium sulphate	52	4940	95.0	65.0
DEAE-cellulose	15	3465	231.0	46.0
Trypsin-Affigel	6.3	2250	357.0	29.5
Sephacryl	2.9	1450	500.0	9.00

The inhibitor units were calculated using bovine trypsin.

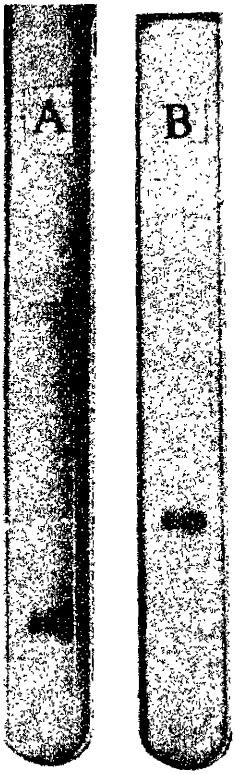

Figure 4. Polyacrylamide gel electrophoresis of purified inhibitor. **A:** In the presence of SDS at pH 7.2; **B.** In the absence of SDS at pH 8.3. (Experimental details are described under materials and methods).

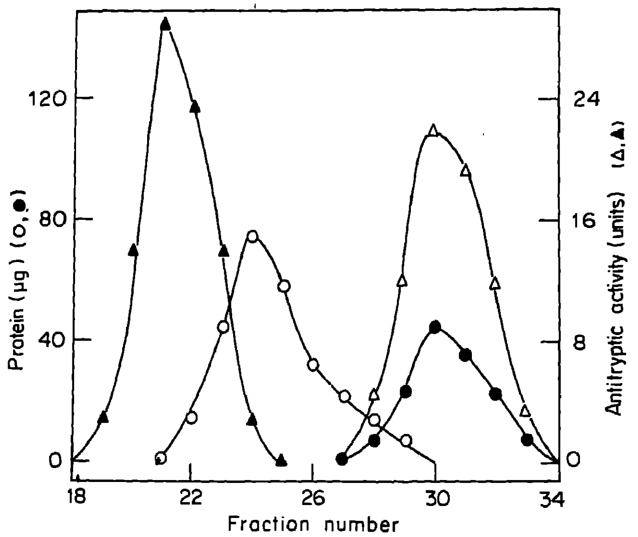

Figure 5. Studies on complex formation between trypsin and purified inhibitor by chromatography on Sephadex G-100.

(Experimental details are described under materials and methods).

(●) Inhibitor, protein; (Δ) Inhibitory (antitryptic) activity; (O) Trypsin, protein; (▲) Trypsin-inhibitor complex, protein.

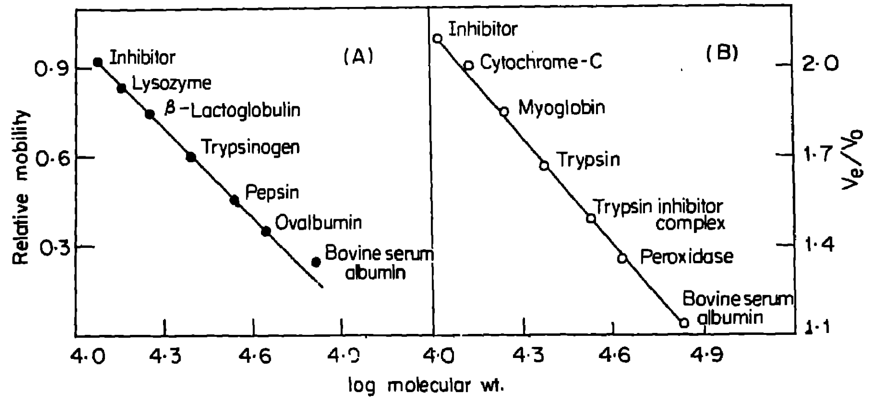

Figure 6. **A.** Molecular weight determination of purified inhibitor by SDS-PAGE.

(Experimental details are given under materials and methods).

B. Molecular weight determination of purified inhibitor and trypsin-inhibitor complex by gel chromatography of Sephadex G-100.

(Experimental details given under materials and methods).

The action of the inhibitor was linear with respect to protein concentration up to 60% inhibition of trypsin and 80% inhibition of α-chymotrypsin. Beyond these levels the inhibition was progressive but non-linear. The inhibitor affected both the caseinolytic and amidolytic activities of bovine trypsin to equal extents. The inhibitor was 1.6 times more active on trypsin than on α-chymotrypsin. This ratio was slightly less than the value of 1.8 obtained with crude extract of arrow root tuber. The purified inhibitor had no action on subtilisin BPN and porcine elastase even when used at 10 times higher concentration of the inhibitor that caused complete trypsin inhibition. The caseinolytic activities of bovine and human pancreatic preparations were equally diminished by the purified inhibitor. However, in both cases the inhibition was not linear beyond 25% and the relative inhibition in terms of caseinolytic activity was much less compared to the action on bovine crystalline trypsin and α-chymotrypsin. The data are summarized in table 2. The purified inhibitor also inhibited bovine enterokinase. The inhibition was linear up to 60% activity. In terms of inhibition of trypsinogen activation, it can be calculated that 1.45 μg of the inhibitor blocked an amount of enterokinase that can activate 4.5 μg of trypsinogen under the assay conditions.

Table 2. Action of the inhibitor on different proteases.

Protease	Inhibitor (μg protein)	Inhibition (units)	Enzyme inhibited (μg)
Bovine trypsin	1.45	0.72	2.4
Bovine trypsin*	1.45	—	2.2
Bovine α-chymotrypsin	1.45	0.45	1.5
Porcine elastase	14.50	0.00	0.0
Subtilisin BPN'	14.50	0.00	0.0
Bovine pancreatic extract	1.45	0.18	—
Human pancreatic extract	1.45	0.17	—
Enterokinase	1.45	—	4.5**

* BAPNA was used as substrate.

** Expressed as amount of trypsin generated under assay conditions.

The purified inhibitor was highly heat stable. No loss of antitryptic activity was noticed on subjecting it to heat treatment at 100°C for 60 min. However, pressure cooking for 20 min, resulted in the complete inactivation of the inhibitor. The inhibitor was also fully stable to exposure to a wide range of pH from 1.0 to 12.5 for 72 h at 4°C. Treatment with pepsin or elastase also did not cause any loss of the inhibitory potency.

Based on the data on the linear range of inhibition it was found that 1.1 mol of the inhibitor could inactivate 1.0 mol of active bovine trypsin. The calculated K_i value for the inhibitor with respect to trypsin was 0.91×10^{-1}M. Complex formation between bovine trypsin and the inhibitor was studied by gel chromatography on Sephadex G-100. The elution profiles of the inhibitor, trypsin and the enzyme inhibitor mixture are shown in figure 5. The single peak observed with the mixture did not display any caseinolytic activity or antitryptic activity, indicating that it represents the inactive complex between trypsin and the inhibitor. The molecular weight of the complex based on its Ve/Vo value was 34,000 which is close to the expected value of 35,000. The complex did not also display any antichymotryptic activity.

The data on the effect of chemical modification of the inhibitor on its antiprotease activity are presented in table 3. Treatment with TNBS or OMI did not affect the antitryptic activity. However, these amino group modifiers caused a partial loss of the antichymotryptic activity. Treatment with CHD resulted in about 50% loss of the antitryptic and antichymotryptic activity. The magnitude of inactivation could not be increased further by increasing the concentration of CHD. Similarly, prior heat treatment of the inhibitor did not make it more susceptible to modification by CHD. Treatment with ninhydrin, another arginyl group modifier resulted in the rapid loss of inhibitory potency. The antichymotryptic activity was slightly more susceptible to ninhydrin than the antitryptic activity.

Table 3. Chemical modification of the inhibitor.

Modifier	Conditions	Antitryptic activity (%)	Antichymotryptic activity (%)
TNBS	24 h	100	67
OMI	24 h	100	75
CHD (500 μg)	3 h	100	100
	6 h	75	70
	16 h	64	60
	24 h	50	45
CHD (2 mg)	24 h	45	45
CHD (500 μg)	24 h, heat treated inhibitor	50	50
Ninhydrin	1 h	22	4
	2 h	19	0
	3 h	0	0

Details are given under materials and methods.

Table 4 represents the data on the effect of preincubation of the inhibitor with trypsin and α-chymotrypsin. Preincubation at pH 3.7 at 37°C or 25°C with trypsin was found to nearly double its antitryptic activity. The time of preincubation in the range 15-60 min did not have any effect on this enhancement. The reaction was temperature dependent, since the magnitude of increase at 4°C was much less. Effect of preincubation at pH 7.6 at 37°C could not be assessed due to a gradual loss

Table 4. Effect of preincubation with protease on the inhibitory activity.

Enzyme	Conditions of preincubation	Inhibitory activity (units)
Trypsin	37°C, pH 3.7, 0 min	0.20
	37°C, pH 3.7, 15 min	0.38
	37°C, pH 3.7, 30 min	0.40
	37°C, pH 3.7, 60 min	0.38
	25°C, pH 3.7, 15 min	0.43
	4°C, pH 3.7, 15 min	0.24
	4°C, pH 7.6, 15 min	0.24
	4°C, pH 7.6, 30 min	0.22
Chymotrypsin	37°C, pH 3.7, 0 min	0.12
	37°C, pH 3.7, 15 min	0.18
	37°C, pH 3.7, 60 min	0.19
	25°C, pH 3.7, 30 min	0.21
	4°C, pH 3.7, 15 min	0.15
	4°C, pH 7.6, 15 min	0.15

of enzyme activity itself in control systems as a function of time. Preincubation with α-chymotrypsin at pH 3.7 also caused an increase in activity.

Discussion

Like the alocasia inhibitor (Sumathi and Pattabiraman, 1977), the arrow root factor acted on both trypsin and chymotrypsin but in addition it also acted on enterokinase. In this respect it is similar to the inhibitor isolated from ground-nut (Lau *et al.*, 1980). Unlike the low molecular weight potato inhibitors (Pearce *et al.*, 1982), the arrow root inhibitor had no action on elastase. The inhibitor was resistant to pepsin and elastase digestion. In addition, it was highly heat stable and also acid- and alkali-stable. In view of these, it could be considered as an antinutritive factor.

Since the trypsin-inhibitor complex had no action on chymotrypsin, it can be concluded that the arrow root inhibitor has a single binding site for the different target enzymes. Chemical modification studies with CHD and ninhydrin suggest that this factor is an 'arginine inhibitor' (Haynes *et al.*, 1967). It has been observed that prior heat treatment of alocasia inhibitor made it more susceptible to chemical modification with CHD (Sumathi and Pattabiraman, 1977). However, this property was not exhibited by the arrow root inhibitor.

It has been reported that when Kunitz soy bean trypsin inhibitor is treated with trypsin at pH 3.7, a single Arg-Ile bond is cleaved resulting in the formation of an active but modified inhibitor (Ozawa and Laskowski, 1966). Similar observations have been made with Bowman-Birk soy bean inhibitor (Siedl and Liener, 1971) and Lima bean inhibitor (Krahn and Stevens, 1972). On the other hand, chick pea inhibitor (Smirnoff *et al.*, 1976) and colocasia inhibitor (Sumathi and Pattabiraman, 1979) were found to lose their activity on pretreatment with trypsin. To the best of our knowledge, increase in antiprotease activity on preincubation of the inhibitor with the protease at pH 3.7 reported here is the first of its kind. Whether this effect is due to the exposure of a second active site needs further scrutiny. It is pertinent to point out that Smirnoff *et al.* (1979) had demonstrated the unmasking of an additional trypsin inhibitor site hidden in the chick pea inhibitor in the trypsin inhibitor fragment isolated after cyanogen bromide treatment followed by digestion with pepsin, of the native inhibitor.

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Studies on triacylglycerol ester hydrolase from bat adipose tissue

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Abstract. Triacylglycerol ester hydrolase was isolated from bat adipose tissue and characterized. The partially purified enzyme had pH optimum of 8.6 and a K_m value of 0.6 mM. The enzyme was denaturated upon freezing and thawing, which was prevented by 25% glycerol. The enzyme was activated by EDTA and NaCl, while it was inhibited by serum and bovine serum albumin. Heparin, sodium fluoride and diisopropyl fluorophosphate had no effect on triacylglycerol ester hydrolase activity. It hydrolyzed triglycerides partially. Triacylglycerol ester hydrolase lost its activity during delipidation but it was reactivated by endogenous lipids and phospholipids, viz. phosphatidyl ethanolamine, phosphatidyl choline and sphingomyelin. The enzyme shows kinetic properties altogether different from lipoprotein lipase and hormone sensitive lipase.

Keywords. Bat adipose tissue; triacylglycerol ester hydrolase; bovine serum albumin; phospholipids.

Introduction

Hormone sensitive lipase [EC 3.1.1.3] and lipoprotein lipase [EC 3.1.1.34] have been extensively studied (Hiromichi and Setsuro, 1974; Mare, 1975; Bolzano, 1977; Setsurp and Yasuyuki, 1977; Anon, 1979). The functional importance of lipase has been shown (Vaughan *et al.,* 1964; Hollenberg, 1965). Matsumura *et al.* (1976a,b) showed the presence of triglyceride lipase in rat and pig adipose tissue in addition to the occurrence of lipoprotein lipase and hormone sensitive lipase. Triglyceride lipase was active in the absence of serum and was strongly inhibited by bovine serum albumin. It was not affected by NaF and cyclic AMP-dependent protein kinase. Similarly many authors have reported hepatic triglyceride lipases in liver homogenates (Varinkova and Mosinger, 1965) and in plasma membrane, cytosol, microsomes and lysomes (Hayashi and Tappel, 1970; Assmann *et al.,* 1973; Teng and Kaplan, 1974; Debeer *et al.,* 1979). The cellular fractions show alkaline pH optima for lipolytic activity, except the lysosomal preparation, which has an optimum of pH 4 to 6.

Very little is known about lipase activity of bat adipose tissue. Geroge and Eapan (1959) assayed lipase in the crude extracts and showed a difference in the levels of the enzyme in brown and yellow adipose tissues of bat. No reports are available on

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Abbreviations used: BSA, bovine serum albumin; DFP, diisopropyl fluorophosphate the isolation and characterization of lipase from the adipose tissue of bat. In the present studies attempts were made to purify partially and characterize the lipase. The enzyme showed kinetic properties altogether different from hormone sensitive lipase and lipoprotein lipase. Hence the enzyme has been referred to as triacylglycerol ester hydrolase or triglyceride lipase (Matsumura *et al.*, 1976a, b).

Materials and methods

Materials

Diphenyl carbazid was obtained from E. Merk, Dermstat, Germany, Diphenyl carbazone was from Veb Jenpharm Laborchemie, Apolda, Germany. Tris (hydroxymethyl)-aminomethane was from British Drug House Ltd., Poole, England. Bovine serum albumin (BSA), phosphatidyl ethanolamine, phosphatidyl choline, sphingomyelin, cholesterol, lysolecitin, phosphatidyl inositol, phosphatidyl serine, triglycerides and brain extract were obtained from Sigma Chemical Company, St. Louis, Missouri, USA. Diisopropylfluorophosphate (DFP) was from Kotch-Light Laboratories Ltd., Colnbrook, Bucks, England. Other chemicals were of the highest purity commercially available.

Methods

Purification of triglycerides from olive oil: Triglycerides from olive oil were purified using neutral alumina gel (Jensen *et al.*, 1966). Purity of triglycerides was checked with thin layer chromatography (Sawant and Varute, 1973).

Preparation of the substrate emulsion: The emulsion of triglycerides was prepared in gum acacia according to Fraser and Nicol (1966). Other emulsifiers when used were dispersed in appropriate buffer for the preparation of substrate emulsion.

Preparation of delipidated tissue powder: The bats, *Cynopterus sphinx sphinx* were collected from their local natural habitat and were killed by occipital blow. The interscapular brown adipose tissue was dissected out and pooled. The tissue (6.4 g) was homogenized with approximately 10 vol of chilled acetone and the mixture was rapidly filtered through Whatman No. 1 filter paper. The residue was washed with 10 vol of chilled acetone and 10 vol of chilled ethyl ether. The defatted residue of adipose tissue thus obtained weighed one g. It was stored below 0°C.

Preparation of enzyme: Delipidated adipose tissue powder was homogenized in 0.1 M Tris-HCl buffer pH 8.6, containing 1 mM DFP, 1 mM EDTA and 25% glycerol. The homogenate was agitated in a metabolic shaker at the rate of 240 strokes per min with 4 cm amplitude for 60 min at 20°C. It was then centrifuged for 30 min at 10000 *g*. The clear supernatant was removed and stored below 0°C. But lipase denatures upon freezing and thawing. Extraction of the enzyme in a medium containing 25% glycerol prevented denaturation and made it possible to store the enzyme at 0-4°C. This enzyme preparation can be stored for several weeks without loss of its activity.

Enzyme assay: The lipase was assayed by the method of Hayashi and Tappel (1970) except for free fatty acids. The assay system contained 0.25 ml of substrate dispersed in gum acacia, 1.0 m. of 0.1 M Tris-HCl buffer pH 8.6, and suitably diluted enzyme sample in a total volume of 1.5 ml. The final substrated concentration was 0.6 mM. Incubations were carried out in a metabolic shaker with continuous shaking at the rate of 160 strokes per min with 4 cm amplitude for 10 min at 37°C. At the end of the incubation the liberated fatty acids were measured colorimetrically according to Itaya (1977).

Protein determination: Protein was estimated by the method of Lowry *et al.* (1951). The protein in wet adipose tissue was measured according to Tornqvist and Belfrage (1976).

Esterase assay: Esterase was assayed with *p*-nitrophenyl acetate as a substrate, as described by Egelrud and Olivercrona (1974).

Enzyme unit: One unit of lipase was defined as the amount of enzyme, which produced one n mol of free fatty acids per minute under appropriate assay conditions (Matsumura *et al.*, 1967a, b).

Results

The levels of lipase activity in wet adipose tissue from different locations are shown in table 1.

Table 1. Levels of triacylglycerol ester hydrolase in adipose tissue from different locations in bat.

Adipose tissue	Enzyme activity Units/g wet tissue	Specific activity
Male (5)		
Interscapular	800 ± 69	15.59 ± 0.77
Epididymal	800 ± 69	15.35 ± 0.90
Female (5)		
Interscapular	860 ± 66	15.16 ± 0.73
Lumbar	1180 ± 88	17.98 ± 0.47

Values in the paranthesis represent the number of animals used for the study.

Bat adipose tissue lipase was denatured upon freezing and thawing, and the denaturation can be prevented by 25% glycerol (Giudicelli and Boyer, 1973). Then the homogenate of adipose tissue in Tris-HCl buffer pH 8.6, containing 25% glycerol was incubated for 30 min at 50°C and the lipase activity was increased by about 35%. The lipase activity was found in the clear supernatant after centrifugation of the homogenate at 10,000 *g* for 30 min. No activity was associated with the lipid fraction. However lipase activity was found in the fat layer, if the homogenate was not incubated at 50°C in the presence of 25% glycerol.

Characterization of lipase activity

Lipase activity in the homogenate of wet adipose tissue and acetone powder resuspension showed a broad pH optimum, while the enzyme extracted as described in the experimental section had a sharp pH optimum at 8.6.

The triglyceride lipase activity was maximal at 37°C. The delipidated tissue powder resuspended in Tris-HCl buffer pH 8.6, agitated for 1 h, centrifuged at 10000 *g* for 30 min yielded a stable enzyme preparation. The increase in the rate of reaction at pH 8.6 measured as a function of increasing amount of substrate, triglyceride emulsion was apparently maximal at 6 mM. However above 6 mM the velocity was decreased. The K_m was estimated to be 0.6 mM.

The insoluble proteins present in acetone powder resuspension might interfere with the triacylglycerol ester hydrolase activity similar to the extraneously added starch particles.

Effect of NaCl, BSA, serum, DFP, heparin, NaF and Na_2EDTA

The lipase was assayed in the presence of 0.2, 0.4, 0.6, 0.8 and 1.0 M NaCl. The enzyme was stimulated 1.5 fold beyond 0.4 M concentration. The enzyme was markedly inhibited by serum (table 2) and BSA (table 3), while DFP, heparin and

Table 2. .Inhibition of triacylglycerol ester hydrolase activity by serum.

Serum (% v/v)	Inhibition (%)
Nil	00
0.2	73
0.4	83
0.6	85
0.8	88
1.0	91
2.0	91

Table 3. Inhibition of triacylglycerol ester hydrolase by bovine serum albumin (BSA).

BSA (% w/v)	Inhibition (%)
Nil	00
0.002	52
0.004	67
0.006	77
0.008	86
0.010	86

NaF had no effect on lipase activity. The activity in the presence of 20, 40, 60, 120 mM EDTA was 133, 170, 200, 218 % of the activity in its absence.

Effect of emulsifiers

Gum acacia, glycerol, BSA, tween 80 and ethanol were used as emulsifying agents for the preparation of substrate emulsion to ascertain their effect on the activity of lipase. It was observed that gum acacia, glycerol and ethanol are suitable emulsifiers to prepare the substrate emulsion required for bat adipose tissue lipase.

Effect of lipids

Emulsions of sphingomyelin, phosphatidyl choline, phosphatidyl ethanol amine, phosphatidyl serine, phosphatidyl inositol, phosphatidic acid, triglycerides, brain extract, glycolipid, gangliosides, cholesterol, cholesterol ester and endogenous lipids were prepared separately using an ultrasonic oscillator. A mixture containing 1 mg delipidated tissue powder and 5 μM of lipids were incubated at 37°C for 30 min. In these mixtures phosphatidyl ethanolamine, phosphatidyl choline, sphingomyelin and endogenous lipids enhanced the lipase activity (table 4), while the other lipids had no effect on enzyme activity. Controls were run to check the phospholipase activity. It was found that the enhancement was not due to phospholipase action on the phospholipids, which were used in the experiments. The effect of phospholipids on the lipase was studied in a glycerol free system.

Table 4. Effect of phospholipids on triacylglycerol ester hydrolase activity in delipidated tissue powder.

Phospholipid	Activity (%)
None	100
Phosphatidyl ethenolamine (PE) (5 μmol)	300
Phosphatidyl choline (PC) (5 μmol)	600
PE+PC (5 μmol)	500
Sphingomyelin	275
Endogenous lipids	200

Each value is the mean of three expts.

The enzyme activity in the absence of lipids is taken as 100%.

Discussion

The increase in lipase activity after incubation of the homogenates of wet adipose tissue in the presence of 25% glycerol for 30 min at 50°C was attributed by Giudicelli and Boyer (1974) to the release of additional enzyme from tissue microparticles. Since the activity observed in the fat layer disappeared upon incubation at 50°C, we conclude that the enhanced activity was due to the release of the enzyme present in the fat layer.

The broad optimum pH range of the enzyme activity in the tissue homogenate and the delipidated crude preparation as compared with the partially purified enzyme indicates the presence of more than one form of the lipase in the bat adipose tissue. Matsumura *et al.* (1976a) showed three forms of lipases in rat epididymal adipose tissue. Similarly Brecher and Kaun (1979) suggested the presence of more than one form of lipase in brain microvesicles.

About 52% of the lipase was lost during delipidation with chilled acetone and ethyl ether. However, extraction of lipase from delipidated tissue powder gave a 10 fold purification. Phospholipids like phosphatidyl choline, phosphatidyl ethanolamine and sphingomyelin activate the lipase in the delipidated tissue powder. Mookerjea and Young (1974) showed the activation of membrane bound UDP-galactose: glycoprotein galactosyl transferase by lecithin. Cytochrome oxidase (Maurel *et al.*, 1978) and palmitoyl synthetase in the brush border of free particulate fraction of chicken intestinal mucosa (Lokesh *et al.*, 1977) are dependent on the phospholipids for maximal activity. In general phospholipid activation is usually associated with the enzymes which are located in membranes (Brierley and Merola, 1962; Jones and Wakil, 1967). There appears to be a tacit assumption by many investigators in this field that phospholipid activation merely reflects the need of the enzyme to be in a hydrophobic environment as would be found in the membrane.

The partially purified enzyme did not show hydrolytic activity towards *p*-nitrophenyl acetate indicating that the hydrolase acts only on long chain triacyl glycerols. This enzyme does not catalyse the hydrolysis of diacylglycerols and monoacylglycerols, but it catalyzes the hydrolysis of triacylglycerols in the presence of diacylglycerols or monacylglycerols.

Partially purified triacylglycerol ester hydrolase studied here, shows kinetic properties altogether different from those of lipoprotein lipase and hormone sensitive lipase, but shows resemblence with the triglyceride lipases of rat and pig (Matsumura *et al.*, 1976a, b). Bat lipase was inhibited by serum and bovine serum albumin and was insensitive to DFP, NaF, heparin and 1 M NaCl, thus differentiating from both lipoprotein lipase and hormone sensitive lipase.

Similarly bat lipase differs from hepatic lipases. Alkaline lipase of rat liver was active in the presence of albumin and was inhibited by NaCl (Assmann *et al.*, 1973) and purified rat liver lysosomal lipase was inhibited by fluoride, ethanol heparin (Teng and Kaplan, 1974) and DFP (Carter Jr., 1967). In contrast to the liver lipases, bat lipase was inhibited by albumin, but it was not affected by NaCl, fluoride, ethanol heparin and DFP.

The mechanism of protection of the enzyme against cold and heat denaturation is unknown. Glycerol induced conformational changes may be responsible for the stability upon freezing and thawing of yeast aldehyde dehydrogenase (Bradbury and Jakoby, 1972).

Benasadoun *et al.* (1974) also used 20% glycerol to protect the lipoprotein lipase against heat denaturation, but they have not studied the effect of glycerol on the kinetic properties of the enzyme. Presently attempts are being made in this laboratory to study the effect of glycerol on triacylglycerol ester hydrolase activity. Similarly studies are being made to find out whether bat adipose tissue lipase is a lipid containing or a lipid requiring enzyme particularly in view of the stimulation of its activity by phospholipids. The physiological role of this enzyme in bat adipose tissue is yet to be elucidated, though it showed seasonal variations (Patil and Sawant, 1982). Purification of triacyl glycerolester hydrolase is also being made.

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Dipicolinic acid as a secondary metabolite in *Penicillium citreoviride*

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Abstract. Synthesis of dipicolinic acid in *Penicillium citreoviride* showed typical kinetics of a secondary metabolite. Its synthesis resumed during idiophase and continued through stationary phase of growth. Total duration of synthesis was 100 h at the end of which its synthesis was arrested. Production of dipicolinic acid by the cells was subject to catabolite repression by glucose and was not subject to end product inhibition by exogenously added dipicolinic acid. Unlike the bacteria, dipicolinic acid synthesis in this mold was highly sensitive to inhibition by calcium ions in the growth medium. Calcium promoted sporulation but dipicolinic acid was not found to be present in detectable amounts in mold spores. Addition of dipicolinic acid and Ca^{2+} completely inhibited its *de novo* synthesis, an effect not observed when calcium was replaced by Mg^{2+} When the mold was grown in the presence of calcium alone, its inhibitory effects on *de novo* synthesis of dipicolinic acid were expressed only after some of this metabolite was first synthesised by the producer cells suggesting that the active feedback inhibitor is probably a Ca: dipicolinic acid complex. It is suggested that over-production of this metabolite is very important to the mold in increasing its survival potential in nature by retrieving the essential minerals from the environment through ligand: metal complex at a time when cells are in the process of dying, so that a proper mineral balance is maintained within the cells.

Keywords. *Penicillium citreoviride;* dipicolinic acid; secondary metabolite; DPA regulation. regulation.

Introduction

Importance of dipicolinic acid (pyridine-2,6-dicarboxylic acid; DPA) in nature was highlighted with its discovery in spore cortex of the bacteria and its possible role in conferring heat resistance to the bacterial spores (Perry and Foster, 1955; Church and Halvorson, 1959). Though the direct involvement of DPA with heat resistance of the spores is doubtful (Hanson *et al.*, 1972), its role in conferring heat stability to the spores following heat treatment is now well recognised and has been shown to be related to its ability to form chelates with metal ions particularly calcium (Murrel *et al.*, 1969; Fukuda and Gilvarg 1969; Aronson *et al.*, 1967; Forman and Aronson, 1972). On the other hand, the same metabolite is excreted

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Abbreviations used: DPA, dipicolinic acid; SM, basal synthetic medium.

by some molds, particularly *Penicillium citreoviride* during growth and its overproduction in these organisms does not appear to have any *prima facie* role in its metabolism (Tanenbaum and Kaneko, 1964). A comparison of the regulatory system operating in the sporulating bacteria and the *Penicillium* should provide a clue as to why the secondary metabolites are excreted by some organisms and not by the others.

Unfortunately, there is conflicting evidence in the literature regarding the metabolic route for DPA synthesis in *P. citreoviride*. Tanenbaum and Kaneko (1964) have presented evidence suggesting α-ketoadipic acid as a key intermediate common to lysine and DPA biosynthesis in this organism and that DPA is formed through cyclisation of diketopimelic acid which, in turn, results from α-ketoadipic acid. Interrelationship of lysine pathway to DPA is quite analogous to that with penicillin biosynthesis which is connected to lysine through the same common precursors (Demain 1966, 1971). Alternative route proposed for synthesis of DPA is similar to that in bacteria and involves condensation of aspartyl-β-semialdehyde with pyruvate to form 2-keto-6 amino-pimelic acid, which, in turn, is cyclised to form 3,5-dihydro-4-hydroxyl-DPA, a hypothetical precursor to DPA (Kanie *et al.*, 1966; Hodson and Foster, 1966). Key intermediates proposed in these pathways are totally different making it difficult to rationalise the mechanism involved in regulation of DPA synthesis (Kalle, 1978). We have, therefore, decided to approach this problem by studying overall DPA synthesis in *P. citreoviride* and factors which affect its regulation with a view to understanding the possible physiological role for DPA in this organism.

Materials and methods

Organism

P. citreoviride strain NRRL 3114 was obtained through courtesy of Dr P. W. Vipond of Unilever Research Laboratory at Port Sunlight, U.K., and maintained routinely on Saboraud's agar slants. It was transferred to fresh slants and grown for 8-10 days till it sporulated completely before transfer to synthetic medium.

Medium

The composition of basal synthetic medium (SM) was as follows: 5 g dipotassium hydrogen phosphate, 1 g sodium nitrate, 1 g magnesium sulphate and 7 g urea per litre of distilled water (pH 7.0). Glucose was added at 100 g per liter unless and otherwise indicated. In experiments where glucose was replenished during growth, a twenty fold concentrate of complete medium including glucose was sterilised and added aseptically to the fermenter as required. In studies on effect of calcium on DPA synthesis, calcium chloride was added to achieve desired concentrations of this metal ion in the medium.

Growth studies

In typical experiments, spores from a dozen slants were resuspended in saline, pooled, washed twice in basal medium SM without carbon source and resuspended to give 10^9 spores per ml. This was used as an inoculum for all growth experiments, which were carried out in 100 ml medium placed in 500 ml flask.

Initial inoculum in growth flasks was adjusted to 10^6 spores per ml and incubated at 30°C on a rotary shaker at 300 rpm. In studies on relationship between growth and DPA synthesis, twenty flasks were incubated at 30°C under identical conditions. At a given time, the contents of two flasks were centrifuged, mycelial mass collected and dried at 110°C to a constant weight. The supernatant fluid was analysed for sugar and DPA after filtering through a millipore filter.

Recovery of DPA from fermentation medium

In studies on product inhibition, the contents of the flask were collected when DPA synthesis was maximum. Mycelial mass was removed by centrifugation and supernatant fluid was filtered through a millipore filter. Filtrate was concentrated in a rotary evaporator under vacuum at 90°C by ten-fold. The pH of the medium was adjusted to 9.0 with NaOH and it was passed through a Dowex-1-acetate column (10 mm × 100 mm). The column was washed with 3 bed volumes of water. The water washing's were pooled and concentrated further by three-fold. This fraction was free of DPA and was used in product inhibition studies.

The DPA on column was eluted with 2 M sodium acetate. Eluates containing DPA were pooled, concentrated and acidified to pH 2.0 with HCl. The DPA which crystallised out of solution was separated by filtration. The DPA crystals were dried on filter paper, redissolved in dilute alkali and recrystallised again as described above. Purified DPA was compared with standard compound (DPA, Fluka A.G. Bucks, Switzerland) with respect to solubility, melting point and ultraviolet spectral characteristics.

Analytical methods

For estimation of DPA in fungal spores, the strain 3114, was grown on a large number of Saboraud's agar plates with or without 100 ppm Ca^{2+} added as $CaCl_2$, for 10 days at 30°C. Spores were collected in a minimum quantity of saline by scraping the growth on agar surface. The suspension was homogenised in a blendor and passed through cotton wool to separate mycelia. Spores in the filtrate were collected, washed in saline with repeated centrifugation at 11,950 *g* in a Sorvall centrifuge. Spores were resuspended in a minimum amount of water, autoclaved at 15 lbs pressure for 15 min., acidified with 1 N acetic acid, and held at room temperature (26-28°C) for 1 h. After centrifugation, DPA was assayed in the supernatant fluid. DPA in culture filtrates and spores was analysed by the method of Janssen *et al.* (1958). Glucose was analysed by the method of Petterson *et al.* (1963).

Results

Kinetics of DPA synthesis in P. citreoviride

The DPA synthesis in *P. citreoviride* followed the typical kinetics of a secondary metabolite (Weinberg, 1970). Its synthesis resumed during idiophase of growth and continued through the stationary phase even after cessation of growth (figure 1). During the initial growth phase, almost all of the sugar was used up for synthesis of cell biomass whereas during the later phase, about 22% (w/w) was utilised for DPA production by the cells. This conversion was increased to 52% (w/w) with

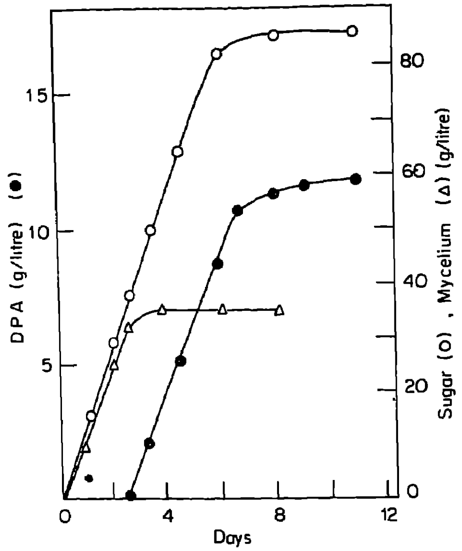

Figure 1. DPA synthesis in *P. citreoviride* in synthetic medium. A three litre batch culture was used with aeration at 30°C; initial inoculum 1×10^8 spores per ml.

concomittant increase in DPA yields by more than two-fold when initial sugar level was decreased from 10% to 5% and maintained at that level through periodic additions till DPA synthesis was completed (figure 2). These results suggest that DPA synthesis is subject to catabolite repression by glucose similar to that observed in case of penicillin biosynthesis (Demain, 1968).

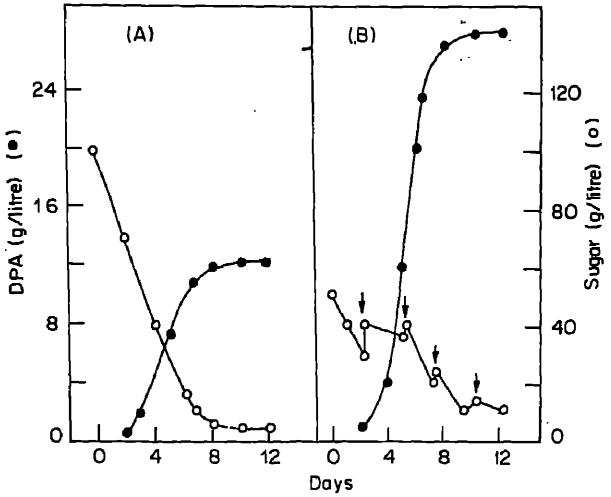

Figure 2. Catabolite repression of DPA synthesis by glucose in *P. citreoviride* grown in synthetic medium. (A) Glucose added at the start of growth—100 g/litre. (B) Glucose added intermittently to maintain concentration at 50 g/litre. Conditions: 100 ml medium/500 ml flask, 30°C rotary shaker at 300 rpm.

Duration and cessation of DPA synthesis

A property of a secondary metabolite is that it is synthesised mostly during a period when there is no further increase in cell biomass. Duration of the synthesis is characteristic of the organism and the metabolite concerned (Weinberg, 1970). In the case of DPA synthesis, this period was about 100 h at the end of which its synthesis was arrested (figure 1). A question may be asked as to why does the synthesis of a secondary metabolite come to a halt. Several possibilities may be considered:

i) Biosynthetic capabilities of the cells may be depleted

Since more than 80% of the total DPA was synthesised during stationary phase of growth, it is possible that mycelia lose their enzyme activities as a part of the degenerative processes associated with the aging of the cells. This hypothesis was tested by growing culture to the stationary phase till DPA synthesis was maximum. The mycelial mass at this stage was 35 g dry weight per litre of the medium. Mycelia were washed twice in fresh medium and resuspended to the same volume. The sugar was monitored regularly and maintained at about 5% level through repeated additions. The DPA continued to be synthesised at the same rate as during the first cycle, but the mycelia lost their ability to produce DPA when recycled for the third time (figure 3). The mycelia, therefore, seemed to retain capability to produce DPA for about 17 days if they were recycled through fresh medium each time DPA levels in the medium reached the maximum value.

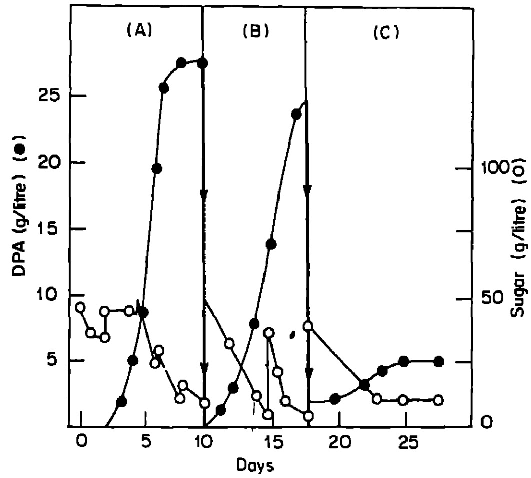

Figure 3. Effect of recycling cells in stationary phase of growth on DPA production. (A) Initial inoculum 1×10^6 spores per ml grown to stationary phase. (B) Mycelia transferred to fresh medium after washing and made upto the same volume. (C) Mycelia transferred to same volume of the fresh medium after washing. Final cell concentration in (A) was 30 g per litre. No further growth in cycle (B) and (C) was observed. Results represent average of 3 flasks.

ii) Feedback inhibition due to accumulation of DPA in the medium

Accumulation of a metabolite (end product) is known to cause inhibition of its own pathway through "negative feedback" inhibition. In order to test if this was responsible, the growth medium was supplemented with DPA at 0.85% and 2%, the latter concentration being near maximum level reached in controls without any exogenously added DPA. The DPA used in these studies was purified from fermentation medium through repeated crystallisations. Results presented in figure 4 indicate that exogenously added DPA did not inhibit its own synthesis. On the contrary, there was a net additional synthesis of 4% when concentration of exogenous DPA was 2% which was twice that observed in its absence. The net DPA formed was isolated from the medium, through repeated crystallisation and its purity was established spectrophotometrically. Results indicate that, instead of feedback inhibition, there was actually a stimulation of *de novo* synthesis in the presence of exogenously added DPA.

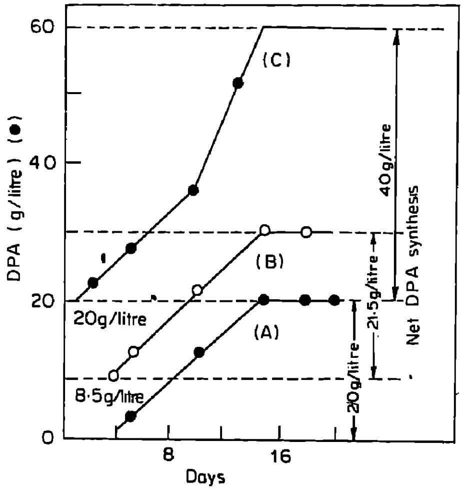

Figure 4. Effect of DPA (exogenous) on net DPA synthesis in *P. Citreoviride*. Initial inoculum 1×10^8 spores per ml. Culture grown in synthetic medium with glucose as carbon source maintained at 50 g/litre by periodic addition and DPA added at (A) 0 g/litre (B) 8.5 g/litre and (C) 20 g/litre.

iii) Inhibition due to products other than DPA

In order to examine the possibility that DPA synthesis in this mold is probably restricted due to the accumulation of inhibitors other than DPA, the strain was grown in the medium till DPA levels reached the maximum (about 2%). After removing the mycelium, supernatant fluid was concentrated ten-fold and DPA removed through crystallisation. The remaining liquor was neutralised and diluted ten-fold in the growth medium so that the concentration of metabolites/inhibitors, if any, would be same as in fermentation medium from which they were originally isolated. The data in figure 5 shows that there is an inhibitory effect which is expressed after about 6 days of growth. Initial rates of DPA synthesis were the same in presence and absence of the inhibitors from the medium but the final levels of DPA reached were only half that obtained in the control.

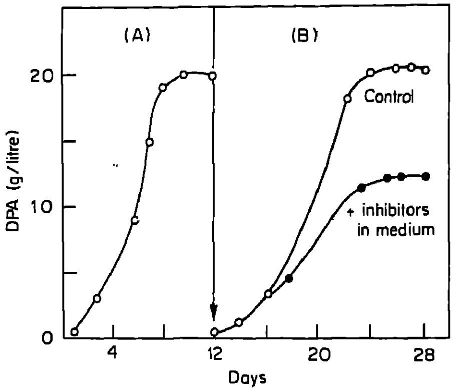

Figure 5. Effect of inhibitors on DPA synthesis in *P. citreoviride*, in DPA-free medium (A) Initial inoculum 1×10^8 spores per ml grown to stationary phase. (B) Mycelia washed and resuspended to the same volume in fresh medium with and without the inhibitor. Final cell concentration in (A) was 32 g/litre. No further growth was observed in (B).

Effect of calcium on DPA synthesis

The DPA synthesis in the mold was highly sensitive to the presence of calcium ions in the medium requiring as low as 5 μM for 50% inhibition (figure 6). As in

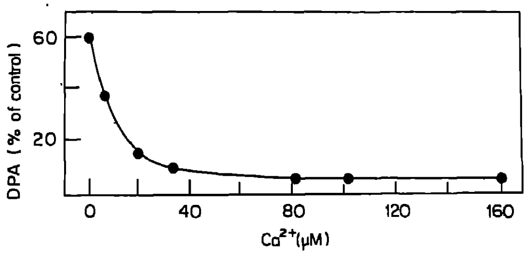

Figure 6. Effect of Ca^{2+} on DPA synthesis in *P. citreoviride*. Calcium added as $CaCl_2$. Concentration of DPA was measured after 6 days of growth. Glucose was maintained at 50 g/litre during growth.

case of bacteria (Murrel 1967; Vinter 1963; Black *et al.*, 1960), the mold showed extensive sporulation both in slide cultures and on plates. One of the explanations for the inhibition of DPA synthesis could be that calcium in this mold promoted sporulation possibly incorporating DPA made *de novo*, thereby depleting the levels of this metabolite appearing in the medium. Data presented in table 1 clearly show that (a) DPA was not detected in fungal spores, (b) addition of exogenous DPA to the medium did not inhibit *de novo* synthesis, (c) addition of calcium (1620 μM) increased sporulation and also suppressed DPA synthesis by more than 90%; however, no DPA was detected in spores, (d) when DPA and calcium were added together at the start of fermentation, inhibition of DPA synthesis was complete, (e) when calcium was replaced by Mg^{2+} there was no inhibition of DPA synthesis, (f) lysine, a primary metabolite which shares a common precursor α-ketoadipic acid with DPA (Demain 1966, 1971) did not inhibit DPA synthesis.

Table 1. DPA synthesis in *P. citreoviride.*

Additives*	Days	Spores (g/100 g cell dry wt.)	Net DPA Synthesis	
			Spores (%)	Medium (mg/g dry wt. cells)
Nil	8	nd	nd	463
	12	nd	nd	868
Lysine (10 g/litre)	8	nd	nd	452
	12	nd	nd	1239
DPA (10 g/litre)	8	nd	nd	445
	12	nd	nd	1177
Ca^{2+} (162 μM)	8	nd	nd	64
	12	56	nd	71
DPA (10 g/litre)+ Ca^{2+} (162 μM)	8	nd	nd	nd
	12	45	nd	nd
DPA (10 g/litre)+ Mg^{2+} (134 μM)	8	nd	nd	380
	12	nd	nd	1080

* All additives added at the beginning of the growth phase.
nd — not detectable.

Calcium in combination with DPA, therefore, appears to be the active inhibitor of *de novo* DPA synthesis, and a partial inhibition observed with calcium alone could be due to the fact that a small amount of DPA had to be synthesised *de novo* before inhibitory effect of the calcium is manifest. An evidence for this view is apparent from results presented in figure 7, where DPA synthesis was monitored at

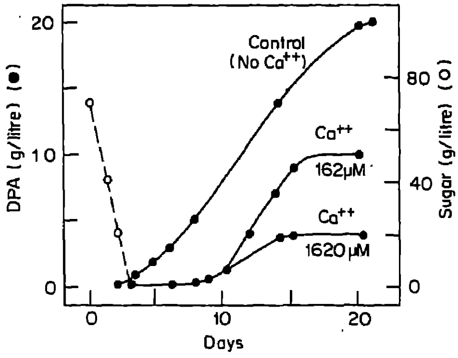

Figure 7. Effect of Ca^{2+} added as $CaCl_2$ on DPA synthesis and sugar utilisation by *P. citreoviride.*

two different levels of calcium. There was an initial delay in the DPA synthesis of about 7-8 days which was common to both levels of calcium. Amount of DPA and its rate of synthesis appeared to be related to the concentration of calcium ions, more DPA being synthesised at 162 μM than at 1620 μM. However, in both cases, certain amount of DPA synthesis occurred before inhibitory effects were finally manifested.

Discussion

Metabolic regulation of DPA in the mould *P. citreoviride* differs from that in bacteria in three important aspects. In bacteria, DPA is not excreted into the medium and its synthesis is controlled through external influence such as calcium and phosphates (Forman and Aronson, 1972, Tamir and Gilvarg, 1974), whereas in this mold it is overproduced presumably due to lack of feedback control over its own pathway. Second important difference is the extreme sensitivity of DPA synthesis in this mold to calcium ion, a phenomenon not encountered in case of bacteria where presence of extracellular calcium helps in the incorporation of DPA in spores.The third point of difference would be that, in bacteria, DPA is present in spores and its physiological role is more or less understood. Whereas in the mold, the DPA is not found in spores and its metabolic role is open to question. In both the systems, lysine is a primary metabolite which shares many of the enzymes common to DPA synthesis. In bacteria, aspartokinase activity which is normally inhibited by lysine, becomes insensitive to inhibition by lysine just prior to the onset of DPA synthesis (Aronson *et al.*, 1967). Data presented here show that DPA synthesis in this mould is not inhibited by lysine or DPA, a fact probably responsible for its over-production.

Extraordinary sensitivity of DPA biosynthesis to calcium is very interesting and seems to be associated specifically with the ability of DPA to form a ligand with calcium. This is evident from the fact that *de novo* DPA synthesis was inhibited by DPA: Ca rather than by exogenously added DPA alone, an effect not observed when Ca^{2+} was replaced by Mg^{2+} (table 1). Similarly, when the mold was grown in the presence of Ca^{2+} and in the absence of added DPA, certain amount of DPA had to be synthesised before its effects became manifest, the amount of DPA synthesised being dependent on the concentration of Ca^{2+} (figure 7). This data suggests strongly that the DPA: Ca is the active feedback inhibitor of the secondary metabolite.

In nature, the physiological role of DPA has been associated with its property to chelate with Ca^{2+} ($pK\,Ca^{2+} - 4.6$). In bacteria, DPA: Ca^{2+} chelate has been considered to be responsible for conferring heat stability to spores (Murrel *et al.*, 1969; Pearce and Fitz-James, 1971). Its synthesis, is therefore closely regulated by external calcium and there is no evidence so far to indicate that the bacteria excrete DPA except during germination. The only recognisible role of DPA : Ca complex in this mold is perhaps to prevent further excretion of DPA by the cells. It would be interesting to speculate that the mold accumulates DPA as a free acid which can enter the cell again only as a metal ligand. The DPA complex with Ca^{2+} is inhibitory to *de novo* synthesis of this metabolite but its complex with Mg^{2+} is not inhibitory. It would thus appear that one of the main roles of DPA synthesis in this mold is to scavenge minerals from the environment at a time when the cells are in the process of dying so that a proper mineral balance is maintained within the cells. This would explain why DPA complex with an essential mineral such as magnesium is not inhibitory. Thus, DPA synthesis is very important to the mold in increasing its survival potential in nature under adverse conditions, somewhat similar to its role in bacterial spores where its function is related to increasing their thermal stability.

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A new bioluminescent fungal system

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Abstract. A new bioluminescent fungal system from a wood sample with a characteristic emission around 518 ± 1 nm is described. This study indicates that water is not only important for emission but has a function in the kinetics of the reaction.

Keywords. Bioluminescence; fungal bioluminescence; *in vivo* kinetics.

Introduction

Bioluminescence is the emission of visible light by a living system. In this process, the chemical energy of an exergonic reaction is converted into light energy via a mechanism in which one of the components of the system is raised to an electronically excited state. Bioluminescent systems (Harvey, 1940, 1952) are quite diverse in nature (e.g. bacteria, fungi, algae, firefly, worms, jelly fish, cypridina, pholas etc). The process of light emission could be continuous (bacteria, fungi) or may occur in brief flashes (firefly and dinoflagellates) and it may be intracellular (bacteria, fungi, firefly) or be extra-cellular with the chemical compounds in the medium in which the reaction occurs (jelly fish).

The word luciferin and luciferase are generic names and are used for the active materials of the luminescent system even now. So far, the luciferin from a limited number of bioluminescent organisms have been isolated and characterised. The luciferin from firefly (Bitler and McElroy, 1957) and cypridina (Shimomura *et al.*, 1957) have been isolated and their structure determined. The cell-free light emission from extracts of bacteria (Hastings *et al.*, 1966) and fungi (Airth and Forester, 1962; Wassink and Kuwabara, 1966; Kuwabara and Wassink, 1966) have also been studied and the mechanism of light emission proposed. The examples of light emission from the so called shining wood have been known for more than a century and is believed to be due to the fungus growing on it (Harvey, 1940). The luminous fungi (Airth and Foerstar, 1962; Wassink and Kuwabara, 1966) known so far radiate in the visible region with a maximum at ≃ 528 nm.

Abbreviation used: IR, infra-red.

We report in this paper, the characteristics of light emission *in vivo* for different samples of the shining wood obtained from different trees which become luminescent during the monsoon, from a village near Lonevala in Maharashtra. Water and oxygen are essential for the emission. Further the emission intensity increases on keeping the wood sample in water for an optimum period.

Materials and methods

The various samples of the luminescent wood studied weighed between 2 to 3 g. The luminescent emission from the samples was recorded with a photomultiplier tube having S-11 response (Type EMI 9514 S). A negative potential of 1000 volts was applied to the photomultiplier tube and the output was recorded on a strip chart recorder through a direct current amplifier. The spectral characteristic of the light emitted by the sample was analysed with a 0.25 meter Jarrell-Ash-Ebert monochromator (model 82-410). The sample was placed in front of the entrance slit of the monochromator and at the exit slit end, a photomultiplier tube having S-20 response (EMI 9558 QB type) was mounted. Light emission spectrum was scanned with the help of a small motor coupled with the grating of the monochromator. The grating motor and the chart movement of the recorder were controlled by a common master switch. The chart speed used was 5 cm/min and the spectrum was scanned at the rate of 100 nm/min. The calibration of the recording unit was first checked with low pressure Hg discharge lamp which gives discrete characteristic mercury lines. The accuracy of the measurement is ± 1 nm.

Results

Intensity of light emission

The intensity of light emission was measured by keeping a small piece of wood dipped under water. The build up and decay curve of the light emission is shown in figure 1. The curve in figure 1 indicates that the emission builds up fast, reaches a maximum value after about 20 min and starts decaying slowly.

Effect of water content on light intensity

A piece of wood sample was soaked with water completely by keeping it under water (water was changed after every 2 days) for 15 days. The sample was taken out from the water container and the light intensity measured. The sample was weighed immediately after the light intensity measurement. The water from the sample was allowed to evaporate with time and periodically the light intensity and the sample weight were measured. A plot of sample weight (wood + water) against the light intensity is shown in figure 2. The data in this figure clearly indicates that the intensity of light decreases with the loss of water from the wood.

Emission spectrum

The emission spectrum of the light from the wood was recorded by the arrangement already described in the experimental part and is shown in figure 3. The emission spectrum in this case is centered around 518 nm.

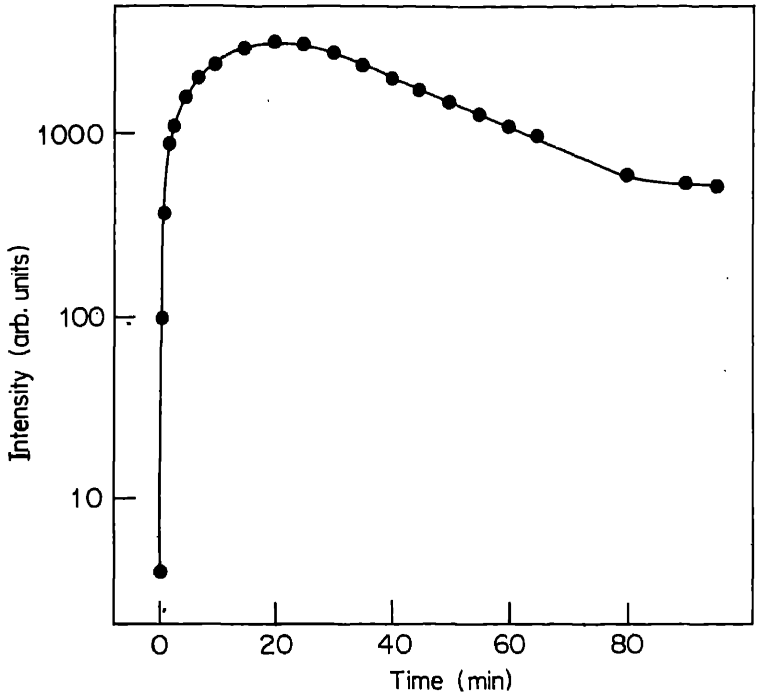

Figure 1. Plot of light intensity versus time when the sample was kept in water during measurement.

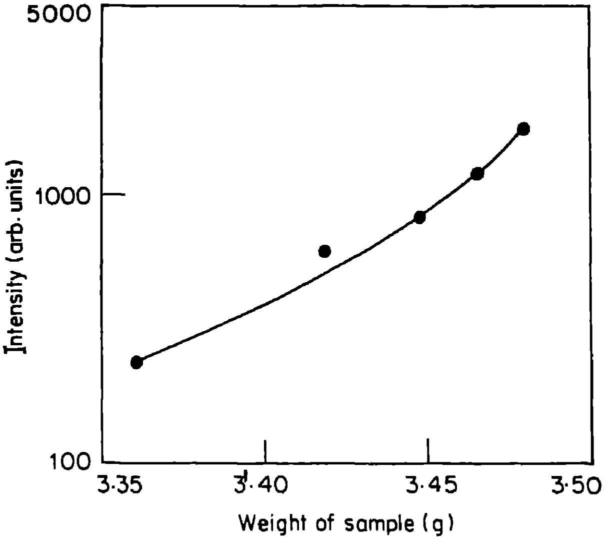

Figure 2. The dependence of light intensity on water content of the sample.

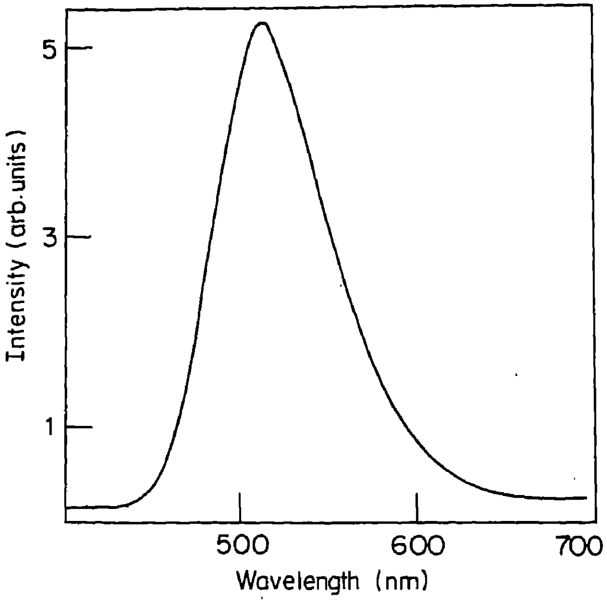

Figure 3. The emission spectrum of the luminous wood.

Effect of nitrogen gas on light emission

During the measurement of intensity of light emission in one of our experiments, nitrogen gas was passed through the chamber containing the sample. The intensity of light emission dropped slowly but could be regained once the nitrogen flow was stopped. The effect of nitrogen flow on the light intensity as shown in figure 4

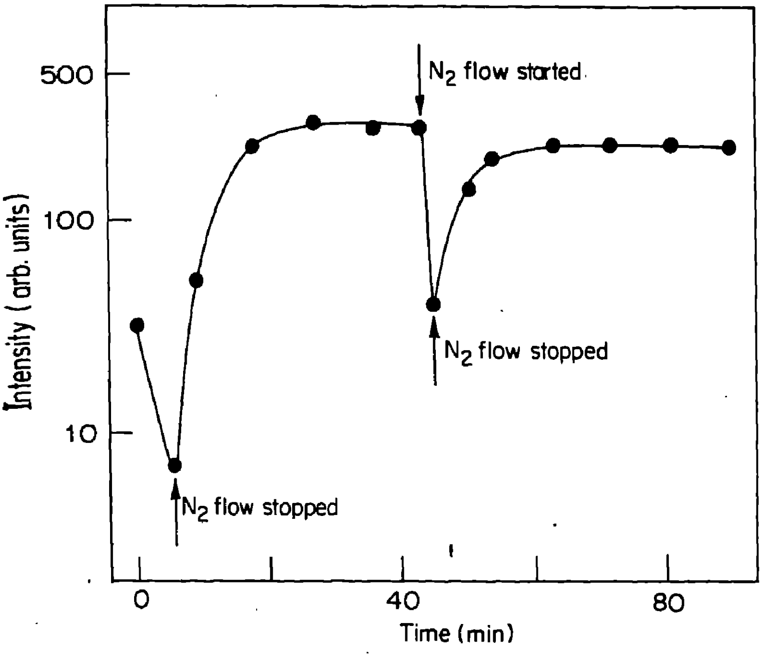

Figure 4. Effect of the presence of N_2 gas on the light intensity.

clearly indicates that light emission requires the presence of oxygen. Secondly the time required to regain the initial intensity depends on the time for which the nitrogen gas has been flowing through the chamber.

Effect of infra-red radiations on light emission

The sample of wood was taken out of water and was exposed to infra-red (IR) radiations from an infra-red lamp (250 w) for two intervals (one after another) of 10·min each. The intensity of light was measured after exposure to IR radiations by keeping the sample dipped under water. The emission intensity decreased with the increasing exposure to IR radiations as shown in figure 5. The results of the figure 5 suggest that the intensity of emission depends on something present on the wood which is sensitive to IR radiations.

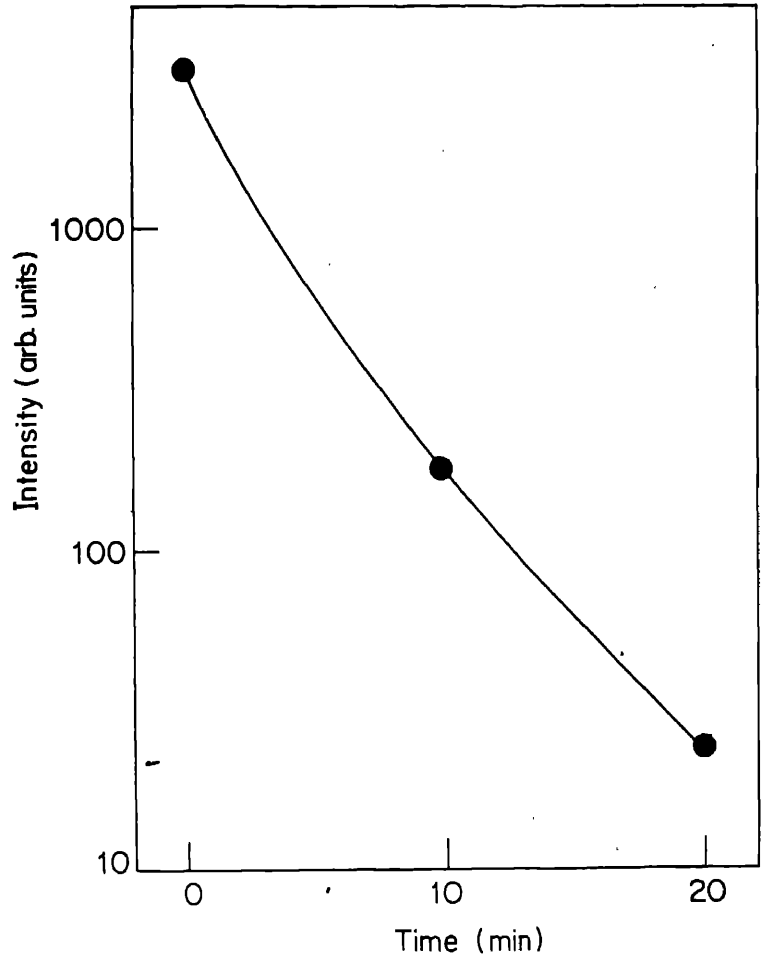

Figure 5. Effect of IR radiations on the light intensity.

Effect of long chain aliphatic aldehyde on light emission

The effect of long chain aliphatic aldehyde (decyl aldehyde) on the light intensity was investigated. For this purpose, during the recording of light intensity, a freshly prepared saturated solution of decyl aldehyde in water at 25°C was poured on to the sample. As a result of this, there was a continual decrease in the light intensity as shown in figure 6.

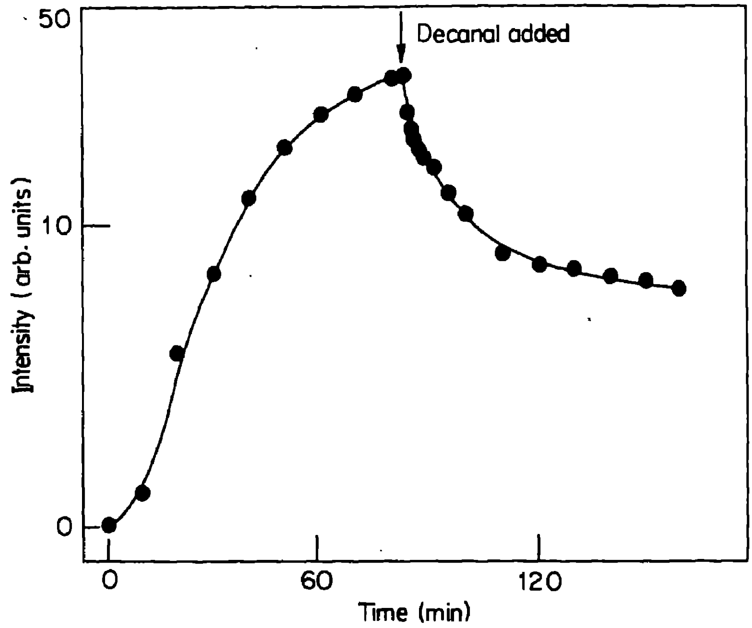

Figure 6. Effect of decyl aldehyde on emission.

Effect of antibacterial antibiotics on light emission

The effect of antibiotics chloramphenicol, actinomycin D and puromycin on the light emission was investigated with the purpose of characterising the nature of the light emitting system. The sample was kept dipped in water containing the above mentioned antibiotics (30 μM) individually and the light intensity was measured as described earlier. None of these antibiotics had any detectable effect on the light intensity over a period of 100 h.

Effect of antifungal antibiotics on light emission

The effect of fungal antibiotics griseofulvin and cycloheximide on light emission was also investigated. For this purpose, the sample was kept dipped in water containing the above mentioned antibiotics (20 μM). The light intensity was found to decrease gradually and after 100 h, decreased to almost to the background level.

In vivo kinetics

The kinetics of the light emission were followed by studying the intensity of light emission as a function of time for the varying contents of water. The water content was varied in terms of the time for which the wood was kept dipped in water. A single sample of almost dried wood (but still weakly emitting) was kept under water for varying intervals of time. The light intensity built up and decay was measured over a constant period of 3 h. The intensity of light was measured after taking out the sample from the water. The period of measurement was not included in the total time of the different readings. The results of these experiments are presented in figure 7. One feature of the data is that the maximum in the light intensity reaches after the wood had been dipped in water for 76 h. Further, the light intensity attains a maximum value in around 20 min irrespective of the time the sample is kept under water. Another prominent feature of the curves in figure 7 is that the time required to increase the intensity is quite short while the decay rate is slower.

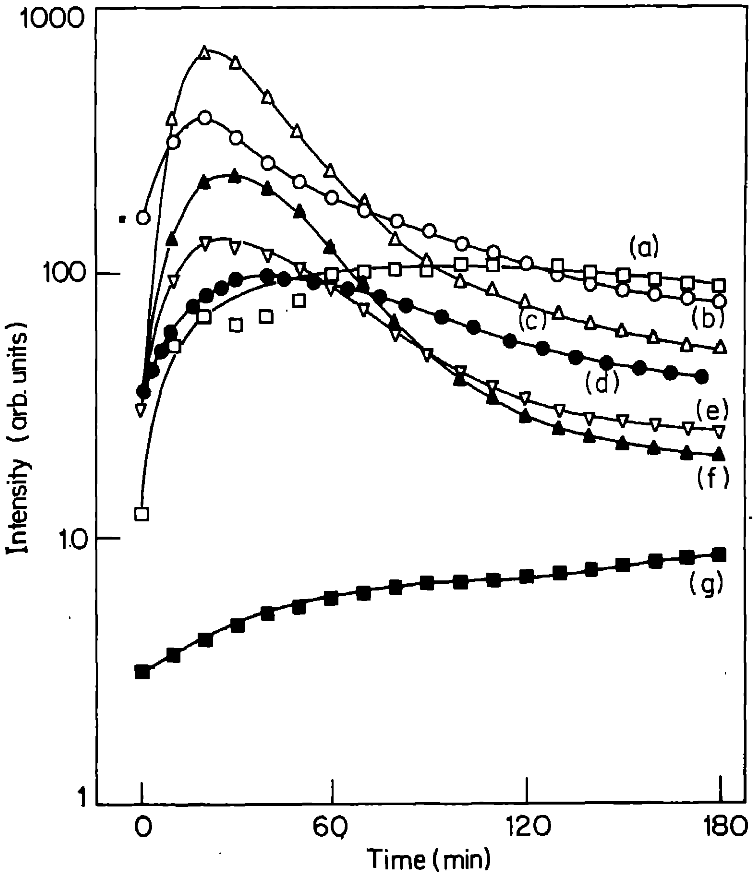

Figure 7. Plot of light intensity versus time for a sample which had been kept in water for different intervals of time before measurements.

a—□ 346 h; b—○ 165 h; c—△ 76 h; d—● 1 h; e—▽ 3 h; f—▲ 8 h; g—■ 10 h.

Discussion

In the present study it was observed that emission could not be revived immediately in a piece of wood which had dried but was emitting weakly. However, the emission could be revived after a few hours if the wood was kept dipped under water. This observation suggests that the cause of the shining wood could be bioluminescent in nature. The bioluminescent emission is also supported by the IR irradiation experiment, since the irradiation with IR decreases the intensity of light indicating the lethal effect of IR radiations on the living organisms. These observations are consistent with those reported by Harvey (1940) for the fungal/bacterial bioluminescent systems. Although these two bioluminescent systems have their characteristic emissions $\simeq 528$ nm (fungal) and $\simeq 490$ nm (bacterial), the general features of their emission are quite similar. Since the maximum in two cases occur at different wavelengths, it is suggested that different molecular species are responsible for the emission. The general nature of the emission spectrum in the present case resembles that recorded by others for luminous fungi or bacteria (Wassink and Kuwabara, 1966). However, the λ_{max} does not correspond to either of these systems. Therefore it is apparently a new bioluminescent system with emission centered around 518 nm. The long chain aliphatic aldehydes are known to have a stimulating effect (Hastings, 1966) *in vitro* on bacterial emission. This effect has been used as one of the indices for distinguishing between the bacterial or fungal system responsible for the luminescence of the wood in the present case. The inhibitory effect of decyl aldehyde (figure 6) *in vivo* suggests that the bacterial system may not be implicated in the light emission from the sample of wood. This was supplemented from the experiment with antibacterial antibiotics which did not have any effect on the intensity of light emission. These two experiments with decyl aldehyde and antibacterial antibiotics suggest that bacteria may not be responsible for luminescence from the shining wood. All these data indicate that the bioluminescence responsible for the shining wood is probably due to a fungus that grows on moist wood. This was confirmed by the inhibitory effect of the antibiotics like griseofulvin and cycloheximide (Cochrane, 1965) which are established fungal antibiotics. This deduction is broadly in agreement with the earlier studies on shining wood (Harvey, 1940) for which fungal system has been determined to be responsible for light emission.

Till todate, the reasons for the shining of wood have been ascribed to luminous fungi predominantly of Basidiomycetes types. Among these the most widely spread is probably the well known wood destroyer *Armillaria mellea.* Three different kind of luminous fungi that have been identified are *Armillaria mellea, Mycena polygramma* and *Omphalia flavida* (Kuwabara and Wassink, 1966). All of these have a characteristic emission in the yellowish green ($\lambda_{max} \simeq 528$ nm) region.

The kinetics of the light emission *in vivo* presented in figure 7 indicates that the light intensity reaches a maximum value in about 20 min after the sample has been removed from water. The maxima in the intensity as well as the decay rate both strongly depend upon the time for which the wood was kept inside the water prior to recording its emission. The peak intensity initially increases with the increasing duration of the sample in water (figure 7, c, d, e, f). The maximum in the intensity of light emission is obtained if the sample was kept in water for 76 h. On further increasing this time, the maximum in the intensity falls continuously (figure 7, a and b). In fact the time dependence of the light intensity for varying contents of water can be divided into two regions (i) the growth region and (ii) the decay region. The growth region indicates that when the wood is kept dipped in water, the living organisms continue to multiply and reach a maximum growth in a period of 76 h. The subsequent decrease in the emission intensity might be either due to the reduction of dissolved oxygen in water or for other unknown reasons. The light intensity is measured after keeping the sample in water in which oxygen is continuously bubbled for 100 h. It is observed that the relative decrease in the emission intensity was roughly twice as large compared to that observed for a sample which is kept in water but no oxygen is bubbled. This clearly establishes that the decrease in light intensity after 76 h is not dependent on the non-availability of dissolved oxygen in water. It might not be out of place to point out the fact that bioluminescent fungal systems in nature are rare compared to nonluminous fungi. This observation is in agreement with that of Foerster *et al.* (1965) who quantitatively measured the light emission from *Collybia velutipes* mycelium of varying age.

The decay region of the kinetic curves can possibly be understood as follows. The wood being opaque, only those emitting centers which are on the surface contribute to the light intensity measurements. The water content in the wood will depend on the amount of time it is kept in water. Initially the total water content in the wood is less. During the recording of the emission, the loss of water takes place by evaporation as has been clearly demonstrated in figure 2, and the water plays an active role in emission process, therefore the light intensity is expected to fall on loss of water. As the total water content in the wood (which increases on keeping it in water for longer time) increases, the loss of water taking place due to evaporation at the surface is compensated to an extent by the diffusion of water from inside the wood to its surface, consequently the emission decays at a slower rate. When the sample is saturated with water, the evaporation and diffusion rates probably match and a very slow decay is observed as in figure 7,a. These results clearly demonstrate that the presence of water is not only essential for detecting the emission but also the kinetics of emission depends upon the total water content present in the wood.

It can be concluded from the present study on the shining wood that a new luminous fungal system with a characteristic emission maximum at 518 nm is present. In order to understand the mechanism of light emission, a detailed study involving the isolation of luciferin and luciferase is required. The culture conditions for the growth of the fungal system for this purpose is in progress.

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Phytochrome regulation of nitrate reductase in wheat

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Abstract. In excised wheat leaves, the activity of nitrate reductase was enhanced by a brief pulse of red light and this increase was reversed by far-red light irradiation. Even under continuous far-red light, nitrate reductase activity increased by 258% after 18 h. When leaves were kept in distilled water during exposure to red light and then transferred to potassium nitrate, there was no difference in endogenous nitrate concentration. The nitrate reductase activity was the same whether leaves were floated in potassium nitrate or in distilled water during irradiation. Partial to complete inhibition of enzyme activity was observed when leaves were incubated in actinomycin-D and cycloheximide respectively, following 4 h of red light irradiation. *In vitro* irradiation of extract had no significant effect on nitrate reductase activity.

Keywords. Phytochrome; nitrate reductase; wheat.

Introduction

Nitrate reductase (EC 1.6.1.1) catalyses the conversion of nitrate to nitrite, and is the first enzyme in the nitrate assimilation pathway. The involvement of phytochrome in the stimulation of nitrate reductase by light in pea was first shown by Jones and Sheard (1972), who suggested that phytochrome controls the enzyme synthesis. Johnson (1976), however, has shown a very rapid induction of nitrate reductase by red light irradiation which was cycloheximide insensitive, thus suggesting activation of preformed enzyme.

Recently, mediation of phytochrome in the regulation of nitrate reductase by light has been shown in the etiolated seedlings of *Sinapsis alba* (Johnson, 1976), maize (Duke and Duke, 1978; Rao *et al.*, 1980), rice (Sasakawa and Yamamoto, 1974), and cauliflower (Whitelam *et al.*, 1980).

In a previous paper, we briefly reported the reversible effects of red and far-red light on nitrate reductase activity in excised wheat leaves (Vijayaraghavan *et al.*, 1979). In this paper, we present detailed experiments to show that in wheat, both low and high energy responses are operative in controlling nitrate reductase by phytochrome.

Materials and methods

Wheat seeds (*Triticum aestivum* var. H.D. 2009) obtained from Indian Agricultural Research Institute, New Delhi, were soaked in running tap water for 24 h and then spread on petridishes for germination in the dark. Leaves were excised from uniformly growing plants on the 7th day. Approximately 250 mg of leaves were taken for each set and floated dorsally in 20 ml of 100 mM KNO_3 solution for induction of enzyme activity.

For enzyme extraction, excised leaves were removed from the nitrate medium, washed with distilled water, blotted dry and ground in 2 ml of extraction buffer containing 100 mM phosphate buffer, pH 7.4 with 1 mM EDTA and 1 mM cysteine, in a mortar and pestle at 4°C, under green safe light. The extract was centrifuged at 18,000 *g* in Janetzki K-24 centrifuge for 15 min. The supernatant was employed for the enzyme assay according to the procedure of Hageman and Hucklesby (1971) with modifications (Sihag *et al.*, 1979). Protein content was estimated following the procedure of Lowry *et al.* (1951) using bovine serum albumin as standard. Nitrate was estimated according to Wooley *et al.* (1960). Green safe light was obtained through 8 layers of green cellophane paper from a white fluorescent tube light. The intensity of green light at the plant level was never more than 1 $\mu W\ Cm^{-2}$. CBS (Carolina Biological Supply, U.S.A.) filters were used to get red (λ_{max} 650) and far-red (λ_{max} 750) light as described earlier (Sharma *et al.*, 1979). Blue light (λ_{max} 450, 140 $\mu W\ Cm^{-2}$) was also obtained through a CBS filter.

Results

Leaves (7 days old) were excised (250 mg) and incubated in 20 ml of 100 mM KNO_3. One set of leaves was kept in continuous white light and two other sets were exposed to 5 min of red and blue light, respectively. The enzyme was assayed after 18 h. Compared to the activity of the enzyme in the leaves kept in the dark, the enzyme activity in the leaves exposed to continuous white light was 494%, in 5 min blue light 55%, and in 5 min red light over 200% (table 1).

Table 1. Effect of red, blue and white light on nitrate reductase induction in 7-day old excised wheat leaves.

Treatment	Specific activity*	Relative activity (%)
Dark 18 h	43	100
5 min red + 18 h dark	94	219
5 min blue + 18 h dark	67	156
5 min white + 18 h dark	94	216
White 18 h	265	594

Red (500 $\mu W\ Cm^{-2}$); Blue (140 $\mu W\ Cm^{-2}$); White light (1200 $\mu W\ Cm^{-2}$).

* n mol of NO_2/mg protein/h.

To check for the involvement of phytochrome, leaves incubated in 100 mM KNO_3 solution were exposed to 5 min of red and far-red light. The enzyme activit increased by 115% on exposure to red light, which was decreased to 43% by far-re light (table 2). However, a second exposure to red light after an initial exposure t

Table 2. Red—far-red reversion of the induction of nitrate reductase activity.

Treatment	Specific activity*	Relative activity (%)
Dark 18 h	59	100
5 min red + 18 h dark	127	216
5 min red + 5 min far-red + 18 h dark	84	144
5 min red + 5 min far-red + 5 min red + 18 h dark	150	254

Far-red light (140 μW Cm^{-2}). * n mol NO_2/mg protein/h.

far-red light increased the nitrate reductase activity to 154%. The reciprocal effects of red and far-red light suggest the possible involvement of phytochrome in the regulation of nitrate reductase in wheat leaves.

To study the kinetics of the enhancement of active red light, leaves exposed to 5 min of red light were transferred to darkness and the enzyme was assayed at different intervals of time upto 24 h. The nitrate reductase activity in dark grown leaves, as shown in figure 1, was detected only after 2 h and after that it increased

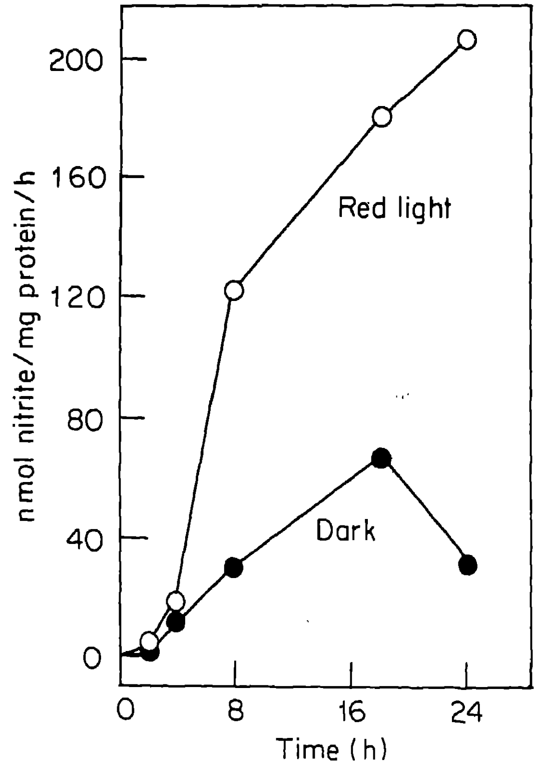

Figure 1. Kinetics of induction of nitrate reductase activity after irradiating the plants with red light for 5 min.

sharply till 18 h, later the activity decreased. In red light irradiated leaves, the lag period was the same as above, but the enzyme activity continued to increase until 24 h.

Even under continuous exposure to far-red light, the enzyme activity increased after a lag of 2 h and reached a maximum value at 18 h, and declined as in the dark control. However the activity was at a higher level (figure 2). This experiment shows that 'high irradiated response' is also involved lending further support to the view that phytochrome may have a role in the control of nitrate reductase.

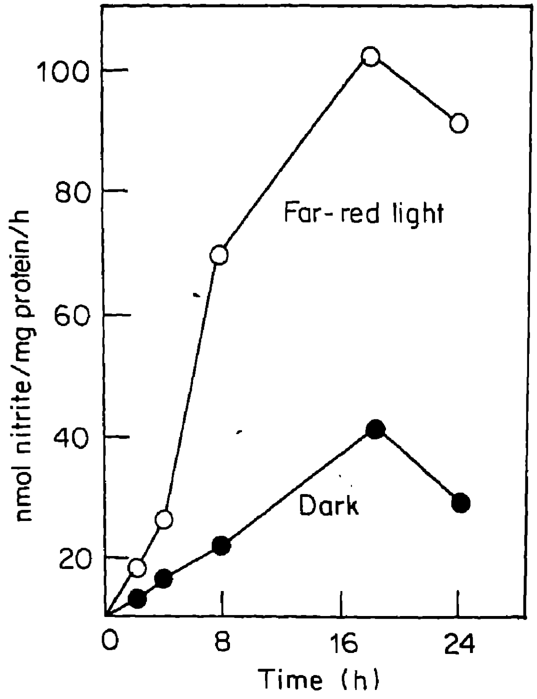

Figure 2. Kinetics of induction of nitrate reductase activity under continuous far-red light irradiation.

To study whether light causes changes in membrane permeability, thus leading to enhanced transport of NO_3^- during illumination, leaves from 7-day old seedlings were excised and incubated either in 20 ml of 100 mM KNO_3, or kept in 20 ml of distilled water, and both the sets were irradiated with red light for 5 min. After irradiation, the plants from both the sets were transferred to fresh medium containing nitrate. As shown in table 3, the leaves which were kept in nitrate during irradiation had the same enzyme activity as those leaves which were irradiated in water. There was also no difference in the accumulation of nitrate in dark and red light treated plants, which was about 312 n mol/g fresh weight.

In another experiment, leaves from 7-day old seedlings were excised and incubated in 100 mM KNO_3 with 20 µg/ml actinomycin-D or 20 µg/ml cycloheximide to study their effect on the increase mediated by short duration of

Table 3. Effect of exposure to red light on the induction of nitrate reductase in leaves kept in water and nitrate medium.

Treatment	Specific activity*	Relative activity (%)
KNO_3 18 h dark	60	100
KNO_3 5 min red + KNO_3 18 h dark	128	214
H_2O 5 min red + KNO_3 18 h dark	124	206

Plants were grown for 7 days in distilled water. On the 7th day leaves were excised and one set was incubated in 20 ml of 100 mM KNO_3 and the other set was kept in 20 ml distilled water and illuminated with red light for 5 min after which from both sets the leaves were trasferred to 100 mM KNO_3 medium. The enzyme was assayed after 18 h.

* n mol NO_2/mg protein/h.

red light treatment. The inhibitors were removed after 4 h of red light irradiation and the leaves transferred to fresh KNO_3 solution. Enzyme activity was measured after 14 h. As shown in figure 3, cycloheximide treatment resulted in 91% inhibition of the enzyme activity, whereas only 32% inhibition was noticed in the leaves incubated in actinomycin-D. This experiment, suggests the probable involvement of RNA and protein synthesis in red light mediated enhancement of nitrate reductase activity.

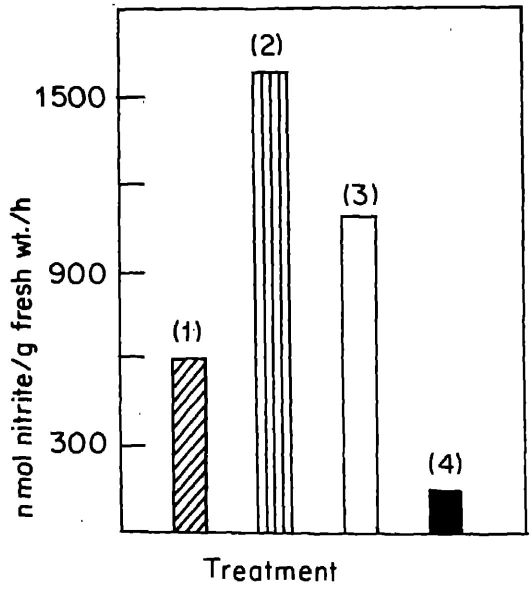

Figure 3. Effect of actinomycin-D and cycloheximide on nitrate reductase activity.

dark; red light for 5 min followed by incubation in the dark for 18 h; red light for 5 min + actinomycin D; red light for 5 min + cycloheximide.

To study if the nitrate reductase is activated directly, the enzyme, after extraction, was irradiated *in vitro*. Also, the leaves (not the extract) were irradiated for 5 min just before the enzyme extraction. When the extract was exposed to red light for 5 min or when the leaves were exposed to red light for 5 min before extraction, about 10-20% increase in the activity was noticed in different experiments in both the cases. As usual, however, 5 min of red light exposure followed by dark interval for 18 h increased the nitrate reductase activity by over 100% in these experiments (data not included).

Discussion

The role of light in the control of nitrate reductase activity has not been fully established, and it is not clear whether such effects are entirely mediated via photosynthesis in chlorophyllous tissue, or involve the phytochrome system. In this work we have not studied the role of photosynthesis but have tried to confirm whether phytochrome regulates the level of nitrate reductase in wheat leaves and to study the mechanism of phytochrome action on nitrate reductase.

The involvement of phytochrome in a system is suggested by (i) low energy response, characterised by red and far-red light reversible phenomenon, and (ii) high energy response. In the present system both these criteria are satisfied. The enzyme activity was increased by a brief pulse of red light, an effect that was reversed by far-red light. When a series of alternating red and far-red light treatments (R+FR+R) was given, the expressed activity of the enzyme was the light given last (table 2). The nitrate reductase activity increased following a lag phase of 2 h.

The involvement of phytochrome in a system is also characterised by the 'high energy reaction' (HER). Here, absolute length of the period of illumination is more important in increasing the enzyme activity. Wheat leaves when kept under continuous far-red light showed an increased enzyme activity upto 18 h following which it declined. There was again a lag period of 2 h (figure 2). Drumm *et al.* (1975) showed that the high irradiation response in anthocyanin formation is related to phytochrome by the use of continuous far-red light. A cyclic phytochrome receptor model explains the action spectrum of high irradiance. According to Schafer (1975), under far-red light treatment, phytochrome reaches a steady state with respect to phytochrome synthesis and phytochrome destruction. The involvement of high energy reaction, which was not reported earlier in wheat confirmed the involvement of phytochrome in this system.

When red light was given to leaves floated earlier on water and then transferred to nitrate or to leaves incubated in nitrate all through (table 3), no difference in the enzyme activity was observed. Moreover, there was no difference in endogenous NO_3^- accumulation ruling out the possibility of increase of enzyme activity due to an increased nitrate uptake by red light.

The presence of a lag phase and the inhibition of nitrate reductase with RNA and protein synthesis inhibitors, actinomycin-D and cycloheximide (figure 3), suggests *de novo* synthesis of the enzyme in this system in response to red light treatment. This is similar to our results obtained in maize (Rao *et al.*, 1980).

Johnson (1976) however, showed that phytochrome induces rapid cycloheximide insensitive increase in nitrate reductase activity in mustard seedlings. When red light was given to the extract or to the leaves just before extraction, although no large stimulation in enzyme activity was observed, thus ruling out the possibility of enzyme activation, 10-20% increase was noticed. It is difficult to envisage the operation of both possibilities—activation and *de novo* synthesis. More work is needed to clarify this point.

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Correlation between inhibition of photosynthesis and growth of *Chlorella* treated with methyl parathion

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Abstract. When methyl parathion (O,O-dimethyl O-*p*-nitrophenyl phosphorothioate), an organophosphorous insecticide, was added to an exponentially growing culture of *Chlorella protothecoides* and the effects were followed for 12 days, the following observations were made: a) In autotrophic culture the cell number and the chlorophyll content decreased as compared to the control. These changes paralleled the inhibition of the rate of net photosynthesis, suggesting that the photosynthetic apparatus was the primary target of the insecticide action. b) The inhibition of cell growth (on cell number basis) also occurred in the case of heterotrophic culture at 100 μM insecticide concentration but the inhibition was less as compared to that of an autotrophic culture. c) The cell diameter in treated culture increased by 10-20% in both autotrophic and heterotrophic cultures. The observations, (b) and (c) suggest that apart from the photosynthetic apparatus, the insecticide has other sites of action, but the sensitivity of these sites to the insecticide is less than that of the photosynthetic apparatus.

Keywords. *Chlorella protothecoides;* methyl parathion; photosynthesis; autotrophic; heterotrophic.

Introduction

Organophosphorous insecticides are widely used in modern agriculture. Very little information is available on the effects of these insecticides on non-target organisms like plants. Recently it has been shown that methyl parathion, one of the most widely used organophosphorous insecticides, inhibits growth of *Chlorella,* a green alga, when the insecticide is present in the growth medium (Saroja *et al.,* 1982). Since in this study the normal cells were inoculated in a medium containing methyl parathion, it was not possible to specify whether methyl parathion affected the metabolism in the lag phase or in the exponential phase. In the present study the insecticide was added to exponentially growing cultures only to avoid greater complexity of the problem. Furthermore, based on earlier observation that methyl parathion inhibits photosynthetic electron transport when added to a suspension of chloroplast thylakoids isolated from higher plants (Anbudurai *et al.,* 1981); in this study we have examined the correlation between inhibition of photosynthesis and inhibition of growth in *Chlorella* upon addition of methyl parathion to an exponentially growing culture of *Chlorella protothecoides.*

Materials and methods

The stock culture of *Chlorella protothecoides* supplied from the algal culture collection of the University of Indiana (ACC No. 25) was maintained in a nutrient medium containing 1g KH_2PO_4, 1g K_2HPO_4, 0.3g $MgSO_4$, 5mg $FeSO_4$, 1 ml of Arnon's A5 solution and 10 µg thiamine hydrochloride per litre, plus 0.1% NH_4Cl as the nitrogen source (Senger *et al.*, 1976). The medium, 140 ml in each of three 250 ml flasks was inoculated with 10 ml of cells from the stock culture and grown autotrophically for 8 days. The flasks were shaken reciprocally at 120 strokes per minute at 25°C under illumination of intensity of 3000 lux. On the 8th day, methyl parathion was added to 2 flasks to obtain concentrations of 100 µM and 200 µM respectively and the remaining one served as the control.

A heterotrophic culture of *Chlorella protothecoides* was maintained in the dark in a medium similar to that for an autotrophic culture, except that the former contained 1% glucose and 0.1% urea. Ten ml of the stock culture was transferred to 140 ml of similar media but containing 100 and 200 µM concentrations of the insecticide and the cultures were grown in the dark. Other procedures were the same as in the case of autotrophic culture.

Cell number was counted with a Neubaur double haemocytometer. Cell diameter was measured microscopically using an ocular micrometer.

For pigment analyses, 10 ml aliquots of the cultures were centrifuged at 2000 *g* for 3 min, to obtain a pellet. Chlorophylls were extracted from cells with methanol, and estimated according to Holden (1967) using the extinction coefficient reported by MacKinney (1941).

Photosynthesis was monitored in terms of the rate of oxygen evolution measured polarographically under saturating light intensity using a YSI 4004 Clark type oxygen electrode (Yellow Spring Instruments Co., Yellow Spring, Ohio, USA) connected to an amplifier followed by a strip chart recorder (Toshniwal Bros., India). The assay mixture contained 4 ml of the fresh nutrient medium containing 10 to 20 µg Chl/ml equivalent of cells. The temperature was maintained at 25°C by circulating water from a thermostated bath around the reaction vessel.

Results

Upon the addition of 100 µM methyl parathion to an exponentially growing culture of *Chlorella*, increase in cell number was slightly inhibited as compared to the initial (zero day) cell number for the following 6 days (figure 1, solid circles). On 7th day onward the cell number started increasing reaching a value of about 20% more than the initial number at the 12th day. Compared to the control (without methyl parathion) culture, however, the inhibition was severe, around 80% on the 12th day. With 200 µM methyl parathion the cell number did not increase, rather a gradual decrease in cell number occurred during the period of observation.

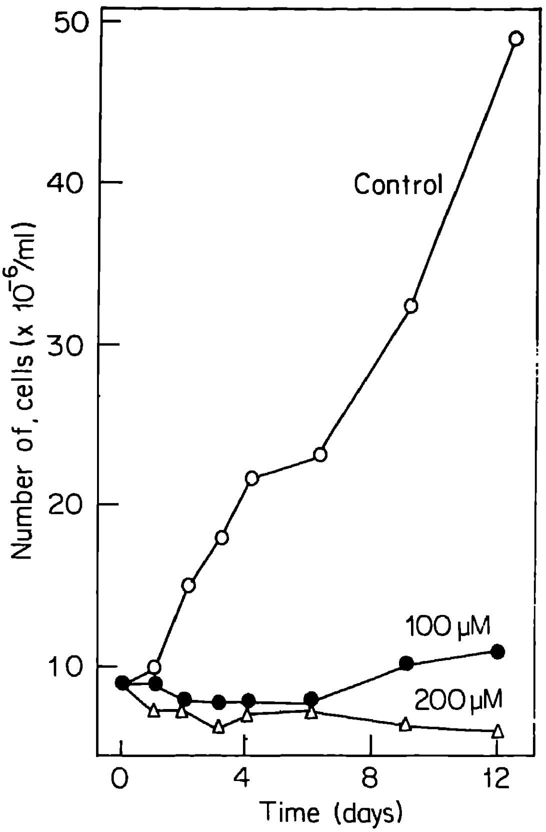


Figure 1. Effect of methyl parathion on cell number of autotrophically growing *Chlorella protothecoides*. Methyl parathion was added to an eight day old culture. The time indicated in the figure is counted from the day of addition of the insecticide to the culture. The inhibition described in the text was calculated using the formula:

$$\%\text{ inhibition} = \left[1 - \frac{\text{Number of cells/ml in treated culture}}{\text{Number of cells/ml in control culture}}\right]$$

Chlorophyll content (per ml of culture medium) decreased during the first 6 days after addition of 100 µM methyl parathion and then showed an increase as compared to the initial value. As compared to the control culture the inhibition was about 80% on the 12th day (figure 2). With 200 µM methyl parathion, the cells were completelv bleached within 5 days after the addition of the insecticide.

Figure 3 shows the rates of photosynthesis as a function of days of growth during treatment with various concentrations of methyl parathion. There was a prompt (in 40 min) inhibition of 25% to 55% depending on the concentration used (figure 3B). The inhibition increased further in a non-linear fashion during the rest of the incubation (figure 3A). With 50 and 100 µM methyl parathion a partial reversal of

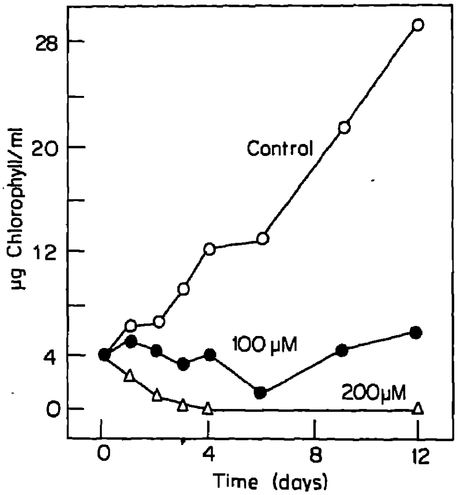
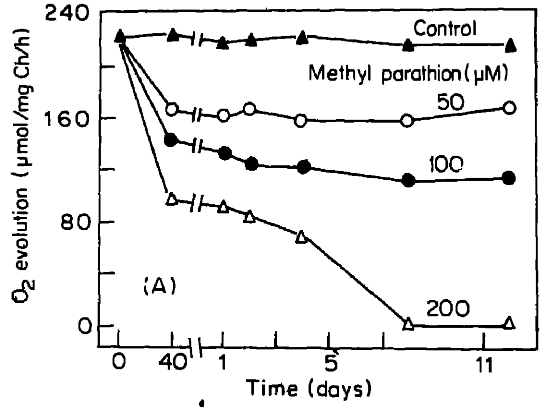


Figure 2. Effect of methyl parathion on total chlorophyll content (per ml) during growth of *Chlorella protothecoides*. Growth conditions were the same as in figure 1. % inhibition was calculated as in figure 1.



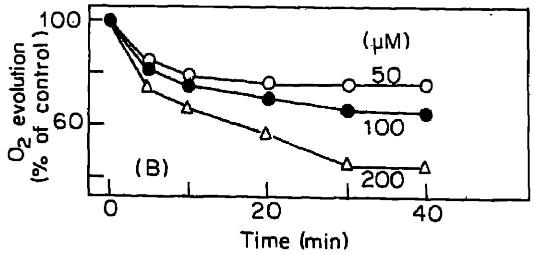


Figure 3A. Effect of methyl parathion on photosynthetic O_2 evolution during the growth of *Chlorella protothecoides*. Growth conditions were the same as in figure 1. Assay conditions for the measurement of photosynthesis have been described in Materials and methods.

Figure 3B. Short-term effect of methyl parathion on the rate of O_2 evolution in *Chlorella*. Methyl parathion at a given concentration was added to a control culture (8 days old) and aliquots were taken at various intervals (as indicated in the abscissa) and the rate was measured.

Control rate of O_2 evolution: 224 µmol/mg chlorophyll. The rate was measured on chlorophyll basis, not on culturre volume basis.

the inhibition was observed, while with 200 μM no O_2 evolution was detectable after 7 days. It should be noted that in control the rate of photosynthesis did not change during the growth period when the rate was measured on chlorophyll basis.

The results described so far showed that all the three parameters (cell number, chlorophyll content, and photosynthesis) measured were inhibited by the insecticide treatment. To examine whether there is any correlation between inhibition of these parameters the data were replotted in terms of inhibition of photosynthesis as a function of inhibition of either cell number or chlorophyll content (figure 4). For a meaningful comparison all the parameters were expressed per ml of culture. A strong correlation between inhibition of photosynthesis and that of cell number (or chlorophyll content) was obtained over a wide range of inhibition measured.

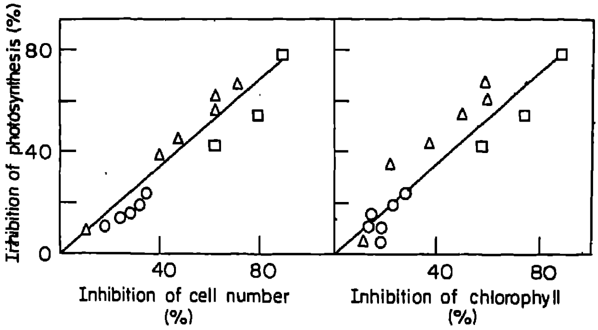


Figure 4. Inhibition of photosynthesis as a function of inhibition of cell number and inhibition of chlorophyll content. Cell number and chlorophyll content were determined from the same batches of cells used for photosynthesis measurements as shown in figure 3. In this figure all the three parameters were expressed on the basis of culture volume and the inhibition was calculated as in figure 1 at various days during the treatment. The circles, triangles and rectangles represent data from treatment with 50, 100 and 200 μM respectively of methyl parathion.

Insecticide-induced inhibition of cell number occurred also in case of non-photosynthetic (heterotrophically grown) cells (figure 5). However, for a concentration below 200 μM the inhibition was greater for autotrophic cells than for heterotrophic cells. For instance, with 100 μM insecticide and at the 5th day, the inhibitions were 80% for the autotrophic cells and 50% for the heterotrophic cells.

Table 1 shows that in treated (100 μM) cells the diameter of the cells increased by about 10 to 20% as compared to the control both in the cases of autotrophic and heterotrophic growth. This increase was outside the normal variability in cell diameter.

Discussion

The parallelism observed between inhibition of photosynthesis and that of cell number (or chlorophyll content) strongly suggests that inhibition of photosynthesis by methyl parathion is primarily responsibile for inhibition of

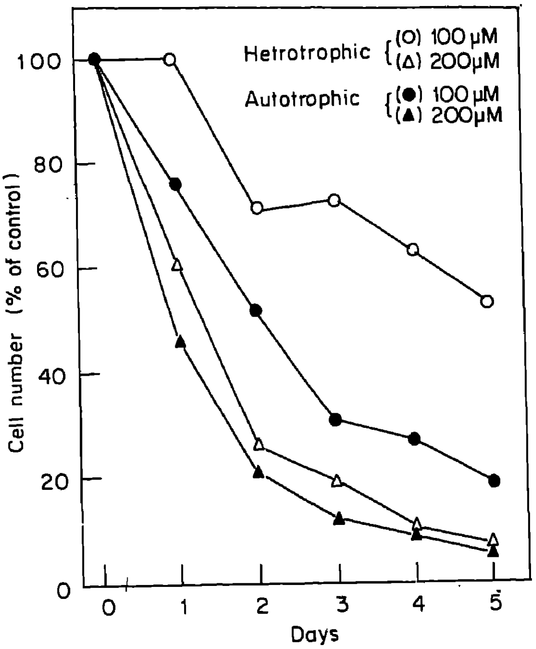


Figure 5. Effect of methyl parathion on cell number of autotrophically and heterotrophically grown *Chlorella protothecoides*. Methyl parathion was added on the day of inoculation. The inhibition was calculated as in figure 1. The cell number at zero time was 1 × 10^6 for control and for treated samples under autotrophic and heterotrophic conditions. The cell numbers (in millions) at 5th day were 20.4 (control), 12.6 (100 µM) and 1.2 (200 µM) for heterotrophic growth, and 8.5 (control), 1.5 (100 µM) and 0.4 (200 µM) for autotrophic growth.

Table 1. Relative cell diameter (as % of control) in the insecticide treated cultures.

Days after addition of methyl parathion (100 µM)	Autotrophic culture	Heterotrophic culture
0	100	100
1	100	103
2	122	109
3	112	116
4	118	113
5	116	109

Each value was the mean of measurements of diameter of 15 cells.

grow[illegible] *Chlorella*. This observation, however, does not exclude other metabolic processes as possible sites of the insecticide action. In fact, an increase in cell diameter during methyl parathion treatment (table 1) suggests that the insecticide affects cell division or related processes.

The prompt inhibition of photosynthesis observed upon addition of methyl parathion in the assay mixture is attributed to inhibition of photosynthetic electron transport, because it has been reported recently that Hill reactions ($H_2O \rightarrow$ dichlorophenol-indophenol or ferricyanide) in thylakoids isolated from Sorghum was inhibited by methy parathion (Anbudurai *et al.*, 1981). The inhibition observed during subsequent days is attributed to superimposed secondary effects, viz., inhibition of chlorophyll content, and changes in the thylakoid membrane organization as indicated by decrease in chlorophyll *a* to *b* ratio (Saroja *et al.*, 1982).

To examine if there are other sites of action, effects of the insecticide has been tested on the growth of non-photosynthetic cells. Inhibition of cell number has been observed in this case also, indicating clearly the existence of other sites of the insecticide action. As expected, however, the magnitude of inhibition is less as compared to the autotrophic counterpart.

The partial reversal of inhibition of growth and photosynthesis observed during the last 4 to 5 days of the experimental period is of special interest. Although methyl parathion degrades rapidly especially within plants, we believe that this reversal was not attributable to the insecticide degradation. Our preliminary studies indicate that an insecticide resistant variant of *Chlorella* develops during the treatment because by transferring the treated cells every few days to fresh media containing insecticide and by continuing this operation for several weeks we obtain cells which are resistant to the insecticide with respect to growth and photosynthetic activity (to be published elsewhere). Reversal of cell growth inhibition occurred also at 200 μM and at higher concentrations of methyl parathion when the treatment was carried for longer periods than reported in this communication.

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Increase in membrane permeability of electrolytes and betacyanin in beet root disc by fenitrothion

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Abstract. Effect of fenitrothion (phosphorothioic acid, 0,0-dimethyl 0-4-nitro-*m*-tolyl ester), an organophosphorous insecticide, on membrane permeability employing the leakage of betacyanin and electrolytes as the criteria were studied in beet root (*Beta vulgaris*) discs. The leakage of both betacyanin and electrolytes increased with increasing concentrations (10-150 ppm) of fenitrothion in the incubation medium. At 0.33 mM the increase in electrolyte leakage was approximately linear for the first 6 h, while the increase in betacyanin leakage started with a lag of about 2 h. Long term incubation (24 h) showed a biphasic nature (in the semilog plot) for the increase in betacyanin leakage, while the increase in electrolyte leakage appeared more complex. In the control sample, the Arrhenius plots (25-50°C) of leakage showed a break at 40°C. In treated samples no break was observed, but the slope decreased (for both electrolyte and betacyanin leakage) as compared to the respective slopes in the control in the temperature region greater than 40°C. The results are discussed in terms of the possible effect of the insecticide on the active transport in plant membranes.

Keywords. Membrane permeability; beet root discs; fenitrothion; betacyanin; electrolyte leakage.

Introduction

In the present day agriculture organophosphorous insecticides are used extensively. The primary target of the insecticides is acetylcholinesterase. Recent studies with liposomes and erythrocyte membranes, however, have shown that these insecticides affect permeability of water, glycerol and proton into these membranes (Antunes-Madeira and Madeira, 1979, 1980, 1981). These observations prompted us to examine if the membrane permeability in plants is also affected by organophosphorous insecticides. To test this hypothesis, we have chosen beet root tissue as a simple plant material and have examined the effects of fenitrothion, a very common organophosphorous insecticide, on the permeability of electrolytes and betacyanin.

Materials and methods

Betacyanin leakage as measured spectrophotometrically and ion leakage measured by conductivity changes were used as criteria in studying the membrane permeability (O'Brein and Prendeville, 1979; Reid *et al.*, 1980). The experimental procedure used was similar to that of Poovaiah (1979) and Poovaiah and Leopold (1976). Fresh beet roots were obtained from a local market and maintained at 4°C until used. Discs of 1 mm thickness and 5 mm in diameter were cut with a cork borer and a gel slicer. The discs were repeatedly washed with double distilled water before incubation. Twenty discs were incubated in 20 ml of double distilled water in a 50 ml beaker at 32°C. Fenitrothion was dissolved in a minimum amount of methanol, and the final concentration of methanol in the incubation medium was less than 1%, and the control received the same amount of methanol. Water baths set up at different temperatures were used for varying the temperatures of incubation. Betacyanin leakage was measured by monitoring the change in absorbance at 540 nm using a Gilford spectrophotometer and ion leakage using a conductivity bridge (MHOS pH meter, Model PE-131, Elico, India).

Results

Effects of concentration

Figure 1 shows the effect of fenitrothion on ion leakage and betacyanin leakage from beet root discs which were incubated for 12 h at room temperature with various concentrations of the insecticide. The values shown in the figure are obtained by subtracting the control values (without fenitrothion) from the values of treated samples. The result shows that the insecticide markedly increased the leakage of both ions and betacyanin. In the concentration range studied the effects were parallel to each other. The apparent difference observed at low concentrations should not be considered, because data obtained from several experiments showed no significant difference between the two effects.

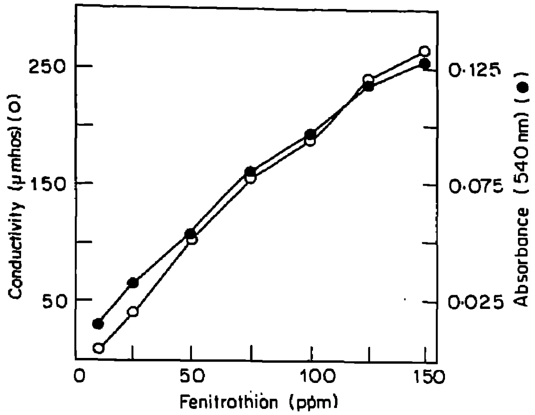

Figure 1. Effect of fenitrothion on the efflux of betacyanin and electrolytes from beet root discs.

Leakage of betacyanin and electrolytes were measured at 12 h after treatment with different concentrations of fenitrothion.

Kinetics of leakage

To examine the kinetics of betacyanin and ion leakage the discs were incubated in solution of 100 ppm of fenitrothion and individual replicates (1 ml) were removed at intervals for the assay of betacyanin. A short term time course upto 6.5 h showed a linear increase in conductivity, whereas there was an initial lag period of 2 h in the case of betacyanin leakage to be observed (figure 2).

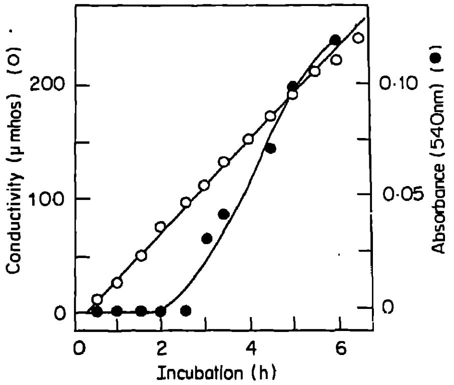

Figure 2. Short term time course of betacyanin and ion leakage from beet root discs.

Beet root discs were incubated in 100 ppm of fenitrothion at room temperature upto 6.5 h. At intervals, the betacyanin and ion content (relative) of the solution were measured. Control values were subtracted from the experimental values at each time interval.

A long-term time course carried out for 24 h with 100 ppm insecticide and data recorded during 3 to 24 h showed, however, an apparent parallelism between the two effects (figure 3). When these data were plotted on a semilog graph, a biphasic nature was revealed with a break at the 12 h, while the ion leakage appeared more complex (figure 3, inset).

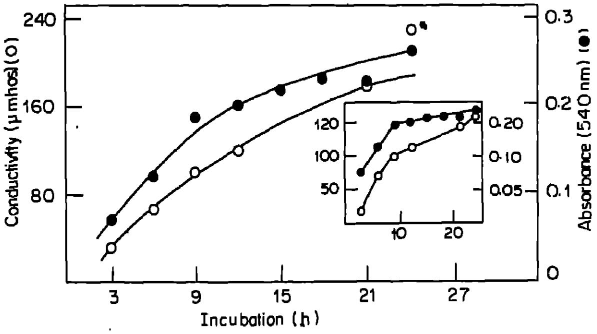

Figure 3. Long term time course of betacyanin and ion leakage. Leakage of electrolytes and betacyanin were measured at 3 h intervals for 24 h with 100 ppm fenitrothion. Control values were subtracted from the experimental values at each time interval.

Inset—Semilog plot of data from figure 3 showing the kinetics of electrolytes and betacyanin leakage.

Temperature dependence of fenitrothion effects

Discs bathed in solutions containing 100 ppm fenitrothion and incubated for 6 h at various temperatures did not show any effect on leakage below 25°C; the effects were observed when the discs were placed in solutions at 25°C and above. Arrhenius plots of conductivity and betacyanin leakage showed a break point at 40°C in the case of control samples (figure 4). Fenitrothion treated samples showed no break in the Arrhenius plots of betacyanin and ion efflux in the

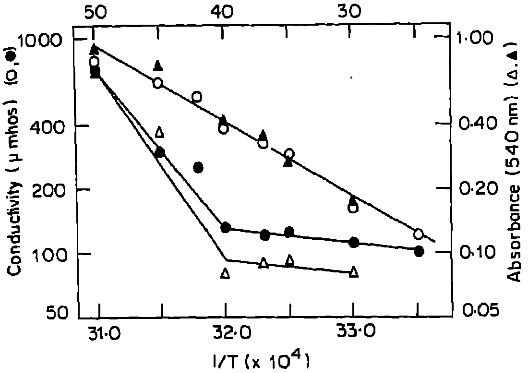

Figure 4. Arrhenius plot of fenitrothion effects on betacyanin and ion leakage.

Beet root discs were incubated in double distilled water with and without 100 ppm fenitrothion at different temperatures (25-50°C) for 6 h treatment.

O, Control; ●, 100 ppm for conductivity; Δ, Control; ▲, 100 ppm for absorbance at 540 nm.

temperature range used. The results also showed a decrease in the slope in case of the treated samples as compared to the respective slopes of the controls.

Discussion

The primary goal of the present study was to find out whether fenitrothion had any effect on membrane permeability in plants. The results demonstrated clearly that permeabilities of both ions and betacyanin in beet root cells were markedly increased. The parallel increase of ion leakage and betacyanin leakage at higher concentrations of insecticide (figure 1) immediately or after prolonged treatment (figure 3) is probably due to non-specific extensive damage that may have occured in the membrane structure.

The observation that there was a lag (~ 2 h) in the betacyanin leakage (figure 2), is not unexpected because both the plasmalemma and the tonoplast membranes should be altered for an effect on betacyanin leakage to be observed, (betacyanin pigments are located inside the vacuole), whereas a change in the plasmalemma alone may affect the permeability of the ions. The biphasic character of the increase in betacyanin leakage is (figure 3 inset) also consistent with the involvement of the two membranes in betacyanin leakage. The complex kinetics of ion leakage is not unexpected, because conductivity is a measure of leakage of ions of various kinds and the effects of the insecticide may not be identical for each ion.

The mechanism of the insecticide action on membrane permeability is not known. The absence of the insecticide effect at low temperature suggests that the passive transport of ions and of betacyanin is not a major site of action of the insecticide at the concentration used (100 ppm) during the period of incubation (6 h). In that case, it will be interesting to examine the insecticide effects on active transport of certain specific ions. A break (around 40°C) in the Arrhenius plots of the control samples suggests that active transport is primarily responsibile for the leakage; the enzymes involved become inactive at about 40°C resulting in an increase in activation energy. In the presence of the insecticide, the activation energy is somewhat lowered as indicated by a decreased slope, and the absence of a break in the line, in the temperature region studied, suggests a shift in the transition temperature beyond this range.

It should be noted that the concentration of fenitrothion and other organophosphorous insecticides used in the solution sprayed in the field is in the range of 100 to 500 ppm (amount used varies between 0.4 to 2 lb per acre); the concentration (100 ppm) used for most of the experiment in the present study is well within this range. This raises a concern on the use of insecticides at the present prescribed doses in terms of plant growth and yield. In fact, recent studies have shown that methyl parathion, another widely used organophosphorous insecticide, decreased the yield (diameter and weight) of lettuce (Sances *et al.*, 1981). A decrease in transpiration and net photosynthesis has also been noted in this report. Saroja and Bose (1982) have shown that methyl parathion (200 to 400 ppm) inhibits severely the growth characteristics of *Chlorella*, and Anbudurai *et al.* (1981) have shown that the rate of electron transport (Hill reaction) in isolated chloroplasts of higher plants is inhibited by methyl parathion with I_{50} around 50 ppm. All these observations suggest the necessity of more careful application of insecticides.

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Suppression of auxin stimulated growth of barley coleoptile sections by endosulfan

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Abstract. Endosulfan, a cyclic sulphurous acid ester commonly used as a broad spectrum insecticide, suppressed the elongation of barley coleoptiles. Indoleacetic acid at optimum concentration overcame the inhibition of growth of coleoptiles treated with 10 ppm endosulfan. However, perfusion of the coleoptile sections with endosulfan and subsequent treatment with indoleacetic acid could not stimulate cell elongation to the extent observed in the control.

Keywords. Endosulfan; indoleacetic acid; cell elongation *Hordeum vulgare.*

Introduction

Germination of *Cicer arietinum* has been shown earlier to be inhibited by prior exposure of the seeds to the pesticide endosulfan (Agarwal and Beg, 1979). Evidence is now presented suggesting interference by the pesticide with the function of indoleacetic acid (IAA).

Materials and methods

Barley (*Hordeum vulgare* Linn) grains were soaked for 10 min in 0.1 (w/v) aqueous hypochlorite solution and washed thoroughly with distilled water. The washed grains were allowed to imbibe water overnight in a refrigerator, spread over moist cotton and germinated at 28 ± 2°C till the coleoptiles attained a length of 1.5 — 2.0 cm. The excised coleoptiles were placed on a glass plate and 1 cm long sections were cut after rejecting 3 mm of the tip portion.

A stock endosulfan solution was prepared in ethanol. Ten ml of either water alone or water containing a predetermined optimum concentration of IAA and 0.05 ml ethanol containing different amounts of endosulfan were taken in petridishes

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Abbreviation used: IAA, indoleacetic acid.

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and coleoptile sections were placed for growth at 30°C for 24 h. In separate experiments, coleoptile sections were infiltrated with endosulfan (10 – 50 ppm) in evacuated Thunberg tubes and incubated for 1 h at 30°C. Controls were taken without endosulfan but with equal volumes of ethanol. After washing thoroughly with distilled water the sections were kept for growth in petridishes containing optimum concentrations of IAA.

The length of the sections were measured using an overhead projector (AIML, India) which gave a seven fold magnification.

Results

The control coleoptile sections showed 40% elongation in 24 h. The endogenous IAA was nearly completely exhausted by incubation of the sections in water for 4 h, i.e. till 15% elongation was observed. The optimum IAA concentration determined was the sum of 1.0 ppm exogenous IAA plus an unknown endogenous level of IAA. This optimum IAA concentration (1.0 ppm) was used in subsequent experiments as IAA-control and the effect of endosulfan was determined at this level. Simultaneously, the effect of endosulfan was also studied against the control. The latter was included to see whether endosulfan alone possessed any auxin like activity.

The data presented in figure 1 show that relatively low level of endosulfan (0.1 – 1 ppm) had no adverse effect on the elongation of the sections. At 10 ppm,

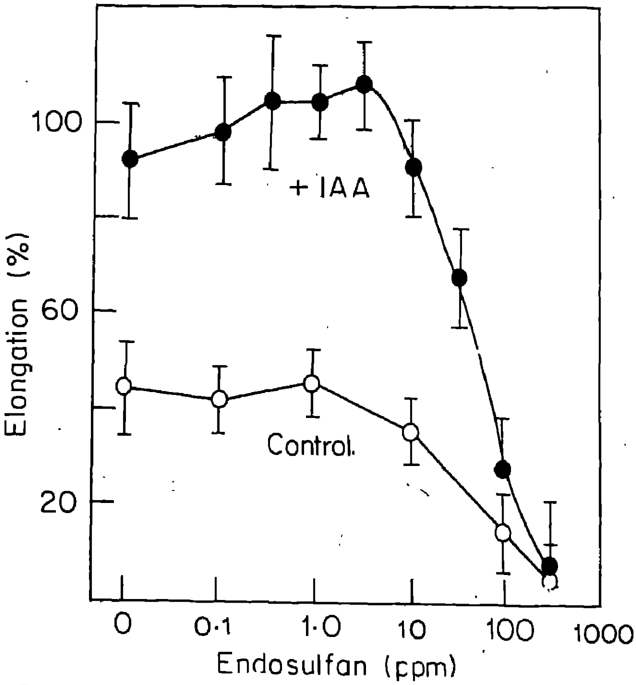

Figure 1. Effect of endosulfan on the elongation of barley coleoptile sections in the presence and in the absence of optimum (1.0 ppm) IAA concentration.

All values are ±SE of 10 observations. Sections were incubated at 30°C for 24 h.

however, a visible inhibition was observed which further increased with increase in endosulfan concentration (figure 1).

The infiltration of excised coleoptile sections with endosulfan at 10-50 ppm caused inhibition of elongation both in water and on supplementation with optimum concentration of IAA (figure 2).

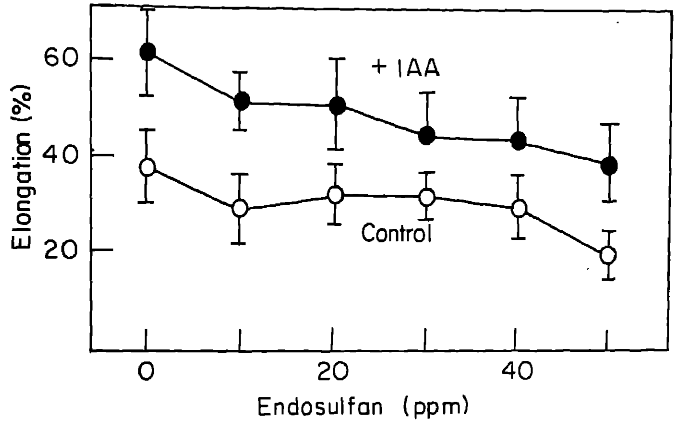

Figure 2. Elongation of endosulfan infiltrated coleoptile sections in the presence and absence of optimum IAA concentration (1.0 ppm).

All values are ± S.E. of 10 observations. Sections were incubated at 30°C for 1 h.

Discussion

The coleoptile growth test is used as a bioassay for IAA activity. Generally, the auxin antagonism or inhibitory properties of xenobiotics are measured at different concentrations against IAA content at maximum growth stimulation. The growth that occurred in the controls is probably due to low level of endogenous IAA remaining in coleoptile sections when the tips (natural source of IAA) were removed. It was evident that all the auxin was not probably depleted and that endosulfan possessed no auxin-like activity within the range 0.1 — 1 ppm. However, the effect of auxin was enhanced insignificantly at this level of endosulfan when IAA and endosulfan were present together.

In the infiltrated barley sections, inhibition of elongation caused by endosulfan was concentration dependent. Barley straight growth test suggests that endosulfan at lower concentrations exerts a competitive inhibition which can be reversed by IAA whereas, at higher concentrations, endosulfan gets irreversibly bound to some site which is needed for the full expression of IAA activity (Hans and Gardner, 1976). Yung and Mann (1967); Lee (1977) and Lau and Yung (1978) demonstrated that metabolites of carbofuran and phenyl carbamate herbicides altered the expression of gibberellic acid and IAA in plants.

The present experimental design gives no evidence for the absence of IAA in coleoptile sectons. The basis for endosulfan inhibition of coleoptile elongation is likely to be the same in the control and in IAA-treated coleoptiles. The data presented indicate that growth inhibition occurs by a similar mechanism, i.e. by auxin antagonism. Our preliminary results indicate that endosulfan antagonizes auxin-stimulated growth which is in agreement with the inhibited growth of *C. arietinum* seedlings on exposure to endosulfan (Agarwal, 1982). Thus, endosulfan exerts its inhibitory effects on seedling growth either by lowering the endogenous level of IAA (Agarwal and Beg, 1982) or by direct interaction with the hormone at its site of action, hampering the hormonal expression.

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Effect of malathion and acetylcholine on the developing larvae of *Philosamia ricini* (Lepidoptera: Saturniidae)

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Abstract. Feeding of malathion induces accumulation of acetylcholine in *Philosamia ricini* developing larvae *via* inhibition of acetyl cholinesterase activity. The insecticide also causes depletion of all nutrients, loss in weight, under-development of silk glands resulting in reduced silk production, lack of oviposition and high mortality among the insects. Acetylcholine however, while fed during the same period of development, tones up their nutritional status, induces better growth, weight gain, improved silk production and oviposition and significantly lower mortality than in the control group of insects maintained on castor leaves. This improved status of insects has been attributed to choline, the insect vitamin released from acetylcholine. Acetylcholine has also been noted to protect the insects to some extent from the poisonous effect of malathion on exposure to it after a dose of acetylcholine during the preceding instar stadium.

Keywords. *Philosamia ricini*; acetylcholine; acetyl cholinesterase; malathion; growth promoter; insecticidal toxicity.

Introduction

Organophosphorus insecticides exert their basic toxicity on the central nervous system *via* inhibition of acetyl cholinesterase by phosphorylating the hydroxyl group of serine in the active site of the enzyme.

Chadwick and Hill (1947) and Smallman and Fischer (1958) confirmed the toxic effect of some lipophilic inhibitors of acetyl cholinesterase in insects and observed a relevant correlation between the sign of poisoning and acetyl cholinesterase inhibition *in vivo*. Thus on the one hand, accumulation of acetylcholine has been shown to poison and induce toxic effects in insects while on the contrary, our observations (Pant *et al.*, 1982) indicate it to be a growth promoter and to some extent as a protector against toxic effects in the lepidopteran phytophagous eri silkworm *Philosamia ricini* when exposed to the chlorofungicide hexachlorobenzene. Therefore, it was considered worthwhile to study the metabolic toxicity exerted by the sulphur-containing organophosphorus insecticide malathion on intermediary metabolism of *P. ricini*, as well as that of acetylcholine-fed insects.

Materials and methods

P. ricini larvae were reared in the laboratory as described earlier (Pant and Agrawal, 1965). At fourth instar stage, larvae were divided into four groups of 500 insects each and lodged in all-round wire-netted wooden cages. Temperature was maintained at 27 ± 2°C and humidity partially controlled as described by Pant and Lacy (1968). Group A was administered acetylcholine, and group M malathion all through fourth and fifth instar stadia, while group B was fed acetylcholine during fourth instar and exposed to malathion in the fifth. The control Group C was maintained all along on *Ricinus communis* leaves.

Before commencing feeding experiments, malathion tolerance was tested on the newly ecdysed larvae through fourth and fifth instar stadia till they pupated. The fatal dose recorded was 90-100 μg/g insect weight/day while the critical dose was 50-60 μg/g insect weight/day.

Malathion and acetylcholine (60 μg each/g insect weight/day) dissolved separately in ethanol were sprayed evenly on fresh castor leaves and exposed to larvae after complete removal of solvent by blowing cold air over the leaves.

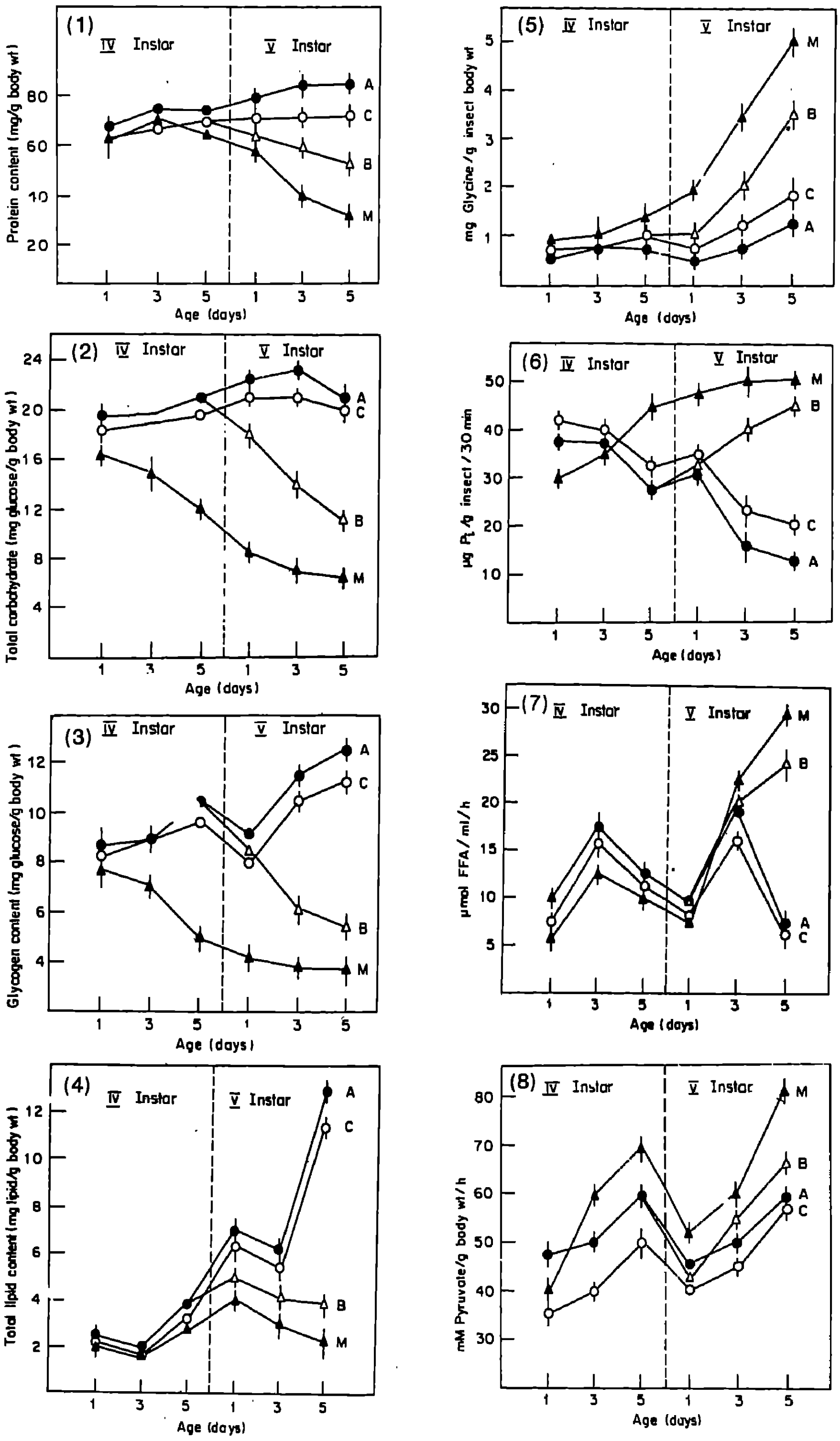
Three lots of 10 larvae each picked randomly from the colony of each group were weighed, chilled in the refrigerator and homogenized to 10% (w/v) tissue concentration in ice-cold glass-distilled water. All assays were carried out in triplicate in each homogenate as well as in the pooled homogenate. The entire experiment was repeated twice over. Although the results did vary quantitatively between 6 and 21%, these variations did not reflect on the variation patterns of the different metabolites and enzymes, observed during development of the insects thus emphasizing the significance of the fluctuations recorded by them.

Proteases were assayed by the method of Matsushita and Iwami (1967) while aspartate (EC 2.6.1.1) and alanine (EC 2.6.1.2) aminotransferases were determined according to Reitman and Frankel (1957). Total free amino acids were estimated by Rosen's method (1957) and proteins by that of Lowry *et al.* (1951). Phosphorylase activity was measured by Green and Stumpf's method (1942), as modified by Srivastava and Krishnan (1961). Lipase activity was assayed by the titrimetric procedure of Roe and Byler (1963). Methods of Ellman *et al.* (1961) was employed to determine the acetyl cholinesterase activity while for the estimation of acetylcholine the method of Hestrin (1949) as described by Metcalf (1951) was adopted. Total lipids were extracted by the methods of Folch *et al.* (1957) and estimated gravimetrically. Total carbohydrates were determined by the method of Trevelyan and Harrison (1952). Glycogen was isolated as described by Wiens and Gilbert (1967) and estimated by the method of Carrol *et al.* (1956).

Each point in figures represents the mean of three observations in triplicate with upper and lower limits. Insignificant variations have not been shown in figures.

Results and discussion

Insects of A group fed acetylcholine all through fourth and fifth instar stadia recorded higher concentrations of proteins, carbohydrates, glycogen and lipids (figures 1A-4A) than those of the control Group C (figure 1C-4C). This was accompanied with lower activity of proteases, phosphorylase and lipases (figures 5A-7A). Aminotransferases (figures 8A, 9A) registered lower activity and



Figures 1-8. (for captions see page no. 92).

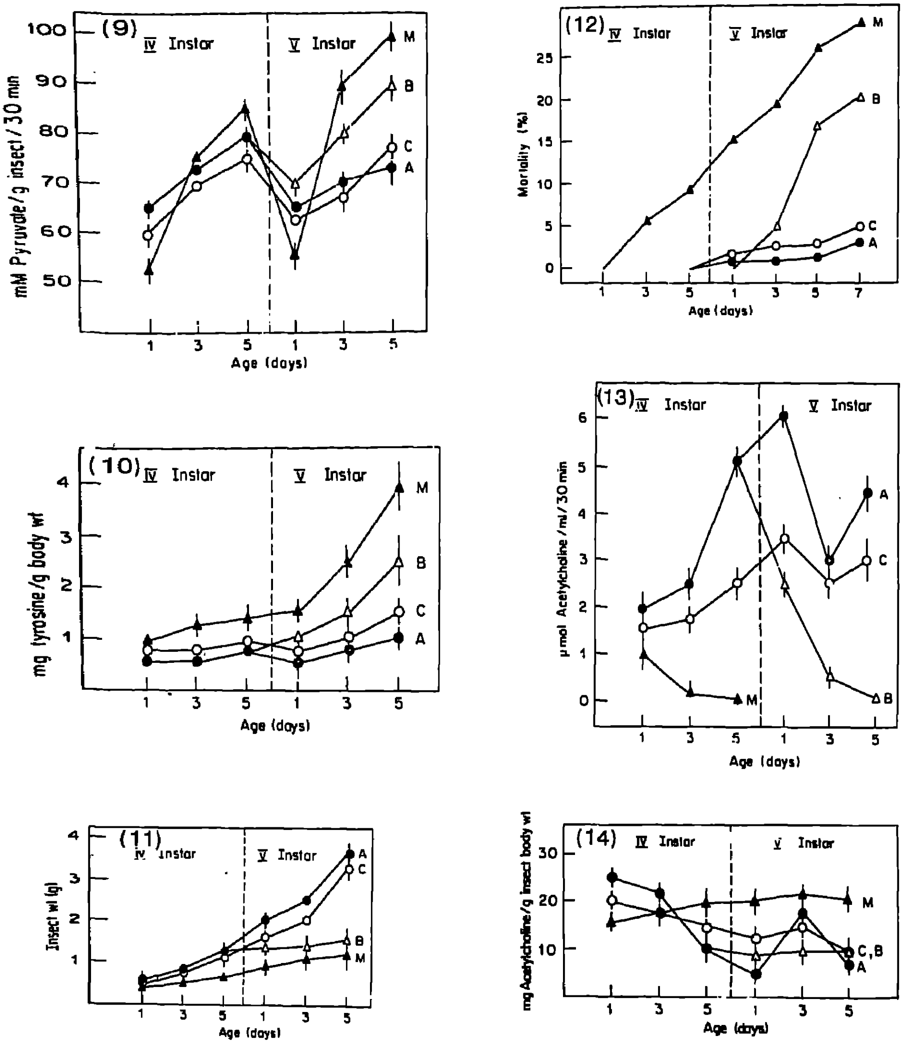


Figure 1-14. The changes in protein (1); carbohydrate (2); glycogen (3); lipids (4); protease (5); phosphorylase (6); lipase (7); aminotransferases (8 and 9); free amino acids (10); weight loss (11); mortality (12); acetylcholine esterase (13) and acetylcholine concentration (14) in acetylcholine and malathion fed *P. ricini*.

accumulated diminished quantities of free amino acids (figure 10A). Feeding of malathion on the other hand, during the same period of development of *P. ricini* larvae, induced opposite and adverse effects. All the afore-mentioned nutrients got depleted significantly (figures IM—4M) with simultaneous enhanced activity of the corresponding enzymes (figures 5M—9M) and increased release of free amino acids (figure 10M). In addition, as recorded in table 1, malathion induced

Table 1. Observations made on the fifth instar developing *P. ricini* larva exposed to various conditions of feeding.

Insect feed	Control (C)	Acetylcholine (A)	Malathion (M)	Acetylcholine followed by malathion (B)
Cocoon wt. (gm)	2.06±0.05	2.24±0.06	1.53±0.10	1.76±0.075
% Cocoon formation	93	94	62	78
Wet weight silk gland (mg)	825±5	841±8	207±6	310±10
Length silk gland (cm)	40±2	40±2.5	25±2	30±3
Length of Larva prior to spinning (cm)	6.0±0.6	6.5±0.4	4.2±0.7	5.0±0.5
Wt. of larva prior to spinning (gm)	3.25±0.15	3.7±0.20	1.2±0.20	1.6±0.10

weight loss (also represented in figure 11M) and stunted growth, under-development of silk glands with impoverished capacity to spin silk and a very high rate of mortality (figure 12M). No moth emergence occurred and those that emerged did not oviposit.

On the other hand, the insects of group B fed acetylcholine earlier and then exposed to the action of malathion appeared to thrive better in every respect (figures 1B — 14B) than the insecticide-fed M group of insects. Mortality rate was also significantly lower (figure 12B). These observations indicate that acetylcholine is protecting the insects to certain extent from the poisonous action of the insecticide. This could be correlated to the acetyl cholinesterase activity (figure 13A) in the acetylcholine-fed group A and acetylcholine concentration (figure 14A) where high activity of the enzyme and low concentration of acetylcholine have been noted. It is assumed that the choline released by acetyl cholinesterase improves the nutritional status of the insects, promotes better growth (figure 11A) and also combats to some extent against malathion poisoning.

Acetylcholine concentrates maximally in group M (figure 14M) than in any other group. The low acetyl cholinesterase activity further declines gradually with development and at fifth instar stage becomes totally inactive (figure 13M) leading to the high accumulation of acetylcholine (22.3 mg/g insect weight, figure 14M). This perhaps results in the loss of muscular co-ordination followed by convulsions and ultimate death of the insects.

The prolonged fourth instar stage by 2-3 days in the insecticide-fed group M suggests disturbance of hormonal equilibrium. Malathion not only destroys insects but also leaves in them a long-standing effect if survived resulting in their destruction by affecting the reproductive function viz., oviposition.

For proper growth and development, choline has been shown as an essential constituent in the larval diet of several insects belonging to several orders and a few species of lepidoptera like the *Bombyx mori* (Horie and Ito, 1965), *Agrotis orthogonia zea* (Kastings and McGinnis, 1967), *Heliothes zea* (Vanderzant, 1968) and *Angyrotaenia velutinana* (Rock 1969) etc. Although *P. ricini* has not yet been experimented upon for its absolute dependence on choline for normal growth, the present investigation provides ample evidence to the fact, that feeding of acetyl choline improves growth, development, oviposition and silk production etc., under normal conditions and during stress, functions as a protector from adverse effects.

The fact that on the one hand acetyl choline proves beneficial to insects and on the other, accumulation thereof in them produces toxicity is rather intriguing. While its accumulation could be due to the inhibition of acetyl cholinesterase activity by malathion, its role as a protector to insects against insecticidal toxicity could be traced to choline released from acetyl choline which tones up the nutritional status of insects.

This speculation is well supported by several other reports (Hayes, 1975; Gains, 1960; Webb *et al.*, 1973) on malathion toxicity that factors like temperature, environment, dosage and mode of exposure of animals as well as the species and their nutritional status modify the toxicity.

Acknowledgement

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Survival of orally administered isolated intestinal *Lactobacillus acidophilus* in different parts of gastrointestinal tract of mice

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Abstract. A strain of *Lactobacillus acidophilus* (Strain HF) was isolated from human faeces. A chloramphenicol resistant strain (HFCm) and a strain (HFCmSm) restant to both chloramphenicol and streptomycin were developed from the isolated strain (HF). All the three strains showed similar *in vitro* susceptibility against host defence factors like gastric acid, bile salts and volatile as well as non-volatile fatty acids. *In vivo* tests were done by feeding these strains to mice. When the resistant strains were orally administered along with the antibiotic(s) they were stable up to 72 h.

Keywords. *Lactobacillus acidophilus;* intestinal lactobacilli; antibiotic-resistant lactobacilli; streptomycin; chloramphenicol.

Introduction

The beneficial role of dietary lactobacilli in the gastrointestinal microecology is now well established (Shahani and Ayebo, 1980). For successful implantation of dietary lactobacilli in the gastrointestinal tract, administration of intestinal lactobacilli strain is essential (Hawley *et al.*, 1959). The species generally used as dietary supplements are . *Lactobacillus acidophilus, Lactobacillus bifidus (Bifidobacterium bifidum)* and *Lactobacillus casei* (Sandine *et al.*, 1972; Speck, 1976). But orally administered lactobacilli are likely to be resisted by host protective factors and by existing intestinal flora. The host protective functions involve the inhibitory effects of the gastric secretion, fatty acids, bile acids and also competition for available nutrients and fermentable sugars (Draser and Hill, 1974; Savage, 1977).

Therefore, for studying survivability and mode of distribution of orally taken lactobacilli in the gastrointestinal tract an intestinal *Lactobacillus* strain was isolated in this laboratory. To differentiate this strain from the lactobacilli existing in the intestine and also to facilitate quantitation, two strains—one resistant to chloramphenicol (Cm) and another resistant to both Cm and streptomycin (Sm) were developed (Bhattacharya *et al.*, 1976, 1977). Susceptibility of these two resistant strains against host defence factors was compared with that of the parent strain and their survivability in the gastrointestinal tract studied.

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Abbreviations used: Cm, chloramphenicol; Sm, streptomycin.

Materials and methods

Isolation of strains

The *Lactobacillus* strain HF was isolated from human faeces where it was a predominant strain. One strain (HFCm) resistant to 50 μg/ml of Cm and another strain resistant to both 50 μg/ml of Cm and 200 μg/ml of Sm, were developed from HF (Bhattacharya *et al.*, 1976, 1977).

Identification of strain

The reference strain used was *L. acidophilus* B-17-1 (obtained from Central Research Institute, Kasauli). Both the strains, reference and the isolated, were tested for growth at 15°C and 45°C (Briggs,1953). Gas production was examined by the method of Rogosa *et al.* (1953). Acid end products from glucose were extracted with ether, and matched with two standard mixtures, one containing 85% lactic acid +15% acetic acid and the other containing 95% lactic acid +5% acetic acid, by thin layer chromatography (Nath *et al.*, 1975). Other tests included motility test, lactic acid configuration (Skerman, 1967) ammonia from arginine, esculin hydrolysis, requirement for thiamine, riboflavin, pantothenate, niacin, folic acid, pyridoxal and Vitamin B_{12}; growth in amygdalin, arabinose, cellobiose, fructose, galactose, lactose, maltose, mannose, melibiose, raffinose, rhamnose, salicin sorbitol, sucrose, trehalose and xylose.

Inoculum

Over night broth culture in de Man-Rogosa-Sharpe medium (deMan *et al.*, 1960) was centrifuged, cell mass was washed thrice and resuspended in sterile 0.9% sodium chloride solution and finally adjusted to 80% transmittance in a Klett-Summerson colorimeter using green filter and used for *in vitro* survival study.

Survival in simulated gastric juice

One ml of inoculum was added per 9 ml of simulated gastric juice (U.S. Pharmacopoeia, 1970) and incubated at 37°C. Solutions having composition similar to simulated gastric juice but without pepsin and conc.HCl were also inoculated and served as control. Aliquots (0.5 ml) were withdrawn at intervals of 30 min, neutralised and serial 10 fold dilutions made in sterile 0.9% sodium chloride solution and 0.05 ml was evenly spread on de Man-Rogosa-Sharpe agar medium. Plates were made in triplicate for each dilution. Colony counts were performed after incubation at 37°C in a candle jar for 48 h.

Bile acid tolerance

Solutions of sodium desoxycholate, cholic acid and sodium taurocholate were made by serial double dilution method and 2 ml from each dilution were incorporated into 18 ml of molten de Man-Rogosa-Sharpe agar medium at 45°C and allowed to solidify. In the case of sodium desoxycholate the pH of the de Man-Rogosa-Sharpe agar medium was adjusted to 7.4. Plates in duplicate were inoculated at 3 sites with 3 different strains. One loopful ($\simeq$0.005 ml) of inoculum was used for each site. Plates were incubated at 37°C in a candle jar for 48 h. Similar plates without any bile solution were prepared and inoculated as above which served as control.

Effect of fatty acids on growth

Molar solutions (4.0 M, 2.0 M, 1.0 M, 0.2 M, 0.1 M and 0.02 M) of fatty acids were prepared and pH of the solution was adjusted to 6.2 ± 0.05. An equal volume (5 ml) of double strength deMan-Rogosa-Sharpe medium devoid of acetate and citrate, and fatty acid solution was mixed. Tubes were made in duplicate and 0.1 ml of inoculum was added to each tube and incubated at 37°C for 24 h in a candle jar. Growth was recorded in a Klett-Summerson colorimeter using green filter.

Animals

Male Swiss mice weighing approximately 25 g were kept in separate cylindrical wire cages (14 × 3 cm) to prevent the movement of the animal and consequently coprophagy and were supplied with sterile pellet food (Hindustan Lever Ltd., Bombay) and sterile drinking water.

Inoculation in mice

The animals were divided into five groups each group containing 12 mice. The first group of animals were challenged twice orally at 12 h intervals with one ml of culture (Ca. 0.5-0.9 $\times 10^{10}$ live cells). The second and third group of animals to be challenged with strain HFCm or HFSmCm were given orally at 12 h intervals, 2 mg of chloramphenicol or 2 mg of chloramphenical plus 4 mg of streptomycin respectively. In the latter case chloramphenicol and streptomycin were given separately. After feeding of the antibiotics, the animals were kept at rest for 2 h, then they were challenged twice orally at 12 h intervals with one ml of culture (Ca 0.5-0.9 $\times 10^{10}$ live cells) containing 50 μg/ml of chloramphenicol or 50 μg/ml of chloramphenicol and 200 μg/ml of streptomycin for strains HFCm and HFSmCm respectively. The fourth group of animals received antibiotic and culture HFCmSm like the third group. Moreover they were supplied orally with one ml of sterile 5% sucrose twice daily starting from 12 h before inoculation and continued up to the end of the experiment. The fifth group of animals obtained antibiotic like the third group but did not receive any culture.

Enumeration of lactobacilli

Freshly voided faecal samples were collected and animals were sacrificied with anaesthetic ether at 8, 18, 24 and 72 h intervals after final administration of culture. Food was withdrawn 12 h before sacrifice. The entire digestive tract was removed and segmented into stomach, small intestine, cecum and large intestine. Each of the segments, including contents from individual animals was weighed, cut into pieces and homogenised in 0.9% sodium chloride solution and several decimal dilutions were made. Faecal samples were also homogenized and diluted as above. A 0.05 ml portion was evenly spread onto the de Man-Rogosa-Sharpe agar plates containing either 50 μg/ml of chloramphenicol or 50 μg/ml of chloramphenicol and 200 μg/ml of streptomycin for enumeration of HFCm or HFCmSm respectively. Plates were made in triplicate, incubated in a candle jar at 37°C for 48 h and colony counts were performed.

Results and discussion

Identification of strain

The strain HF produced 95% lactic acid and no gas from glucose, did grow at 45°C but not at 15°C (table 1). The strain therefore best fitted in the thermobacterium

Table 1. Some important characters of isolated and reference *Lactobacillus* strains.

	*L. acidophilus**	*L. helvetious**	*Strain HF*	*L. acidophilus B-17-1*
Gram character and morphology	Gram-Positive rod occurred in pairs and short chains.	Gram-positive rod occurred in pairs and short chains.	Gram-positive rod occurred in pairs.	Gram-positive rod occurred in pairs.
Growth at 15°C	−	−	−	−
and 45°C	+	+	+	+
Gas from glucose	−	−	−	−
Proportion of lactic acid in comparison to acetic acid	85% or more	85% or more	more than 85%	more than 85%
Lactic acid configuration	DL	DL	DL	DL
Requirements for:				
Folic acid	+	−	+	+
Niacin	+	+	+	+
Pantothenate	+	+	+	+
Pyridoxal	−	+	−	−
Riboflavin	+	+	−	+
Thiamine	−	−	−	−
Vit B_{12}	−	−	+	−
Growth in:				
Cellobiose	+	−	+	+
Melibiose	+	−	+	+
Salicin	+	−	+	+
Sucrose	+	−	+	+
Esculin hydrolysis	+	−	+	+

* As reported in Bergey's Manual — 1974.

group of the homofermentative lactobacilli. The strain produced DL-lactic acid. Only *L. acidophilus* and *L. helveticus* of the homofermentative lactobacilli produced DL-lactic acid.

The strain HF resembled *L. acidophilus* in its requirement for pantothenate, niacin and folic acid, and in not requiring thiamine and pyridoxal and differed in being B_{12} dependent and riboflavin independent. It differed from *L. helveticus* with respect to riboflavin, folic acid, pyridoxal and Vitamin B_{12} requirements. Moreover, the strain HF also closely resembled *L. acidophilus* than *L. helveticus*, being cellobiose, melibiose, sucrose, salicin and esculin positive (table 1). In case of other tests performed there was no difference among *L. helvetious*, *L. acidophilus*, HF and *L. acidophilus*—B-17-1 strains. The strain was therefore designated as *L. acidophilus* HF.

Survival in simulated gastic juice

The viable count of the strains HF and HFCm in simulated gastric juice at 0 h were 8×10^7 and 7.2×10^7, but after 5 h of incubation at 37°C the same were found to be 6×10^7 and 3×10^7 respectively and with strain HFSmCm the reduction of count was very much like the parent strain. As these antibiotic resistant strains were very much like parent strains with respect to their acid tolerance, they would not undergo destruction in the stomach if administered orally.

Bile acid tolerance

Among the bile acids tested sodium desoxycholate was less inhibitory (5.0 mg/ml). But minimum inhibitory concentration was 1.25 mg/ml for all three strains for both cholic acid and sodium taurocholate. The inhibitory effect of bile acids against certain bacteria in the intestine, particularly the Gram-positive bacteria over the Gram-negative bacteria was demonstrated previously (Binder, 1975, Floch *et al.*, 1971, 1972). The present study indicated that the development of resistance against antibiotics did not make the strains more susceptible to bile acid than the parent strain.

Effect of fatty acids on growth

In the basal media containing no formate, acetate, propionate, butyrate, citrate and lactate, Klett reading was around 300 to350 for the parent strain (HF) and antibiotic resistant strains (HFCm and HFSmCm). Effects of above mentioned inhibitors on the growth of these three strains were almost similar. Citrate stimulated the growth up to 0.05M, thereafter growth was inhibited very sharply. In the case of formate and butyrate, a gradual inhibition of growth was observed with increase in concentration and ultimately it reached to zero level at 0.5M concentration. Growth remained more or less steady with increase in concentration up to 0.1M in case of acetate, propionate and lactate but with further increase in concentration growth decreased markedly. Butyrate was found to exert greater antibacterial activity than acetate and propionate. These observations are in agreement with earlier report (Lee and Gemmell, 1972). Since no difference in the inhibitory effect of fatty acids was observed between the parent and the antibiotic resistant variants, the latter strains should not encounter any difficulty in their passage through the intestinal tract.

Survival in gastrointestinal tract

Because orally administered lactobacilli could not be differentiated from the lactobacilli already present in the intestinal tract, the former strains were made resistant to antibiotics. Besides, antibiotic resistant strains could be counted accurately from a mixed population. The *in vitro* survivability studies with these antibiotic resistant strains against some host defence factors as mentioned above indicated that they were as resistant as the parent strain against these agents. Therefore, *in vivo* survivability studies were conducted with these strains to ascertain their distribution in different parts of the intestinal tract.

In the control group (5th Group) where chloramphenicol and streptomycin were administered but not the strain HFSmCm, no significant number of organism could be detected at any time during 72 h. At 8 h after administration, the population of HFCm (figure 1A) found in the stomach and small intestine was 10^3/g (approx) wet wt. while 10^7/g (approx) wet wt. was noted in caecum, large intestine and faeces. The strains could not be detected after 72 h of administration in the stomach and small intestine, whereas in caecum, large intestine and faeces a

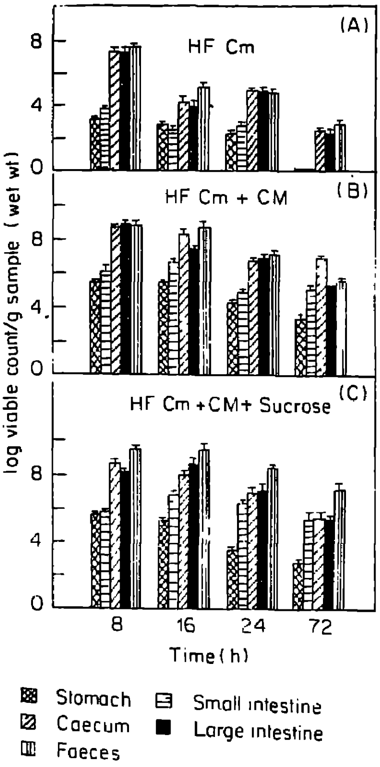

Figure 1. Survival of the strain HFCm in different regions of the gastrointestinal tract and in faeces of mice under: **A.** Normal condition; **B.** Treatment with chloramphenicol; **C.** Treatment with chloramphenicol and sucrose. The vertical bar represents standard deviation.

population of 10^3/g (approx) wet wt. was obtained. The population of the strain HFSmCm (figure 2A) found in the stomach was 10^3/g (approx) wet wt. at 8 h, while it was 10^5/g (approx) wet wt in small intestine. In caecum, large intestine and faeces counts greater than 10^6/g wet wt. were observed. At 72 h no viable count was observed in stomach, small intestine and caecum. In large intestine and faeces population of 10^3 (approx) and 10^4/g (approx) wet wt. respectively were noted. When the mice received antibiotic (figure1B), the population of the strain HFCm found at 72 h in stomach, small intestine, caecum, large intestine and faeces were around 10^3, 10^5, 10^7, 10^5, and 10^5/g wet wt. respectively. Over the same period the population of HFSmCm (figure 2B) found in these habitats under similar condition were about 10^5, 10^5, 10^7, 10^9 and 10^9 respectively. The viable counts of the resistant strains in mice when fed with antibiotic and sucrose (figures 1C and 2C) were found to be more or less same, in different regions of the digestive tract and in faeces, as those observed in mice treated solely with antibiotics.

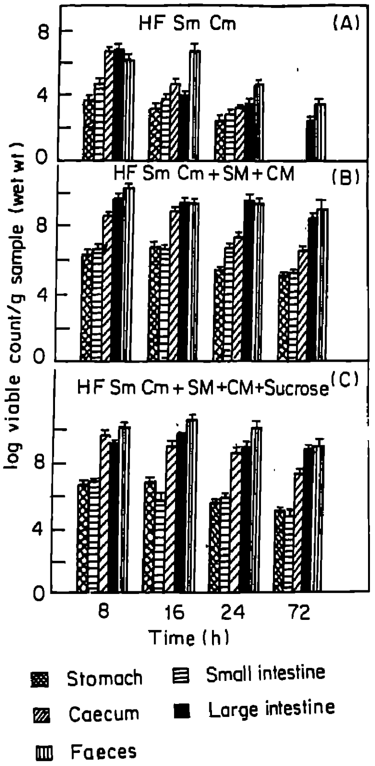

Figure 2. Survival of the strain HFSmCm in different regions of the gastrointestinal tract and in faeces of mice under: **A.** Normal condition; **B.** Treatment with streptomycin (SM) and chloramphenicol (Cm); **C.** Treatment with chloramphenicol, streptomycin and sucrose. The vertical bar represents standard deviation.

It appeared from *in vitro* studies that the survival of the resistant strains would not be hindered by the inhibitory agents of the host's digestive tract. But *in vivo* survival studies of the resistant strains showed that they could not survive in the intestine. Thus, it appears that apart from inhibitory agents like gastric HCl, bile acids, volatile fatty acids, some other factors are involved in the establishment of the strains in the intestine.

The local immunity of the gastrointestinal tract plays an important role in eliminating microorganisms from that site. Allochthonous microorganisms like *Vibrio cholerae*, temporarily colonize the epithelial surface of the small intestine and induce antibody formation. These antibodies prevent increases in population of *V. cholerae* in the murine stomach. But autochthonous microbes prove to be poorly or totally without capacity to induce antibodies, particularly when they are present in the gastrointestinal tract. It is stated that, at least, the surfaces of these autochthonous microorganisms that contact the cells of gastrointestinal tract must be sufficiently related to the host's antigens so as to render them recognisable as "self" by the host's immunological system (Savage, 1977). *Lactobacillus acidophilus* used in the present study being an autochthonous strain, its elimination by the host's immunological mechanism is improbable.

Survival of the resistant strains improved on feeding antibiotics simultaneously. This may be due to the fact that the treatment with antibiotics diminished the intestinal microbial population thereby lessening the resistance of host flora and favouring the growth of invading antibiotic resistant bacteria. This is also supported by the fact that treatment of mice with antibiotics showed increased susceptibility to infection with *Salmonella* (Meynell and Subbaiah, 1963) or *Shigella* and in the case of guinea pigs also to *Vibrio cholerae* (Copper, 1959; Freter, 1956). Hirsh *et al.* (1974) also found that treatment with large dose of tetracycline (1000 mg/day taken orally) favoured the establishment of a tetracycline resistant strain of *Escherichia coli* of bovine origin in the enteric tract of man compared to subjects receiving no antibiotic or a low dose (5 mg/day) of antibiotic. Further improvement in respect of survivability was not obtained on supplementation of sucrose with antibiotic.

In the present studies, orally administered lactobacilli strains of intestinal origin did not flourish in the intestinal tract although they could overcome host defence mechanisms. It may be mentioned that the movement of the digestive tract is a very prominent mechanism for driving out allochthonous microorganisms or food matter. But for autochthonous microbes like lactobacilli it may not be so important, since, the latter strain may attach to epithelia via polysaccharides or glycoproteins and it is stated that *Lactobacillus* strain only of mammalian origin can attach to the epithelium of mouse (Clarke and Bauchop, 1977). It may be presumed that once the lactobacilli are attached to epithelium, clearance by peristalsis will be difficult. In the present study, the invading lactobacilli, possibly, were not able to attach themselves to the epithelial cells as these sites were already occupied by indegenous microbes. Therefore, it appears that, for establishment of orally given lactobacilli in the intestine, suppression of existing intestinal microflora by some inhibitors is an important factor. But one should not interpret the present data solely on the basis of microbial interference at this stage.

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Inhibition of avian myeloblastosis virus reverse transcriptase by an RNA-binding protein from plasma membranes of normal and tumor cells

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Abstract. Purified plasma membranes from normal rat liver, a rat hepatoma and a rat hepatic fibrosarcoma have been shown to contain a protein which drastically inhibits avian myeloblastosis virus reverse transcriptase activity. The inhibition is caused by the binding of the protein to the template. The binding and the consequent inhibition of enzyme activity are template-specific; copying of RNA templates is inhibited whilst that of DNA templates remains unaffected. Investigations using different templates suggest that the inhibitory protein has a stronger binding affinity for G, C-rich templates. The inhibitor appears to have a wide distribution in plasma membranes from diverse sources.

Keywords. Reverse transcriptase; RNA-binding protein; plasma membranes.

Introduction

During the course of a study of the role of host factors in the infection of cells by retroviruses, we have isolated and partially purified a 120,000 dalton protein from plasma membranes of chicken embryonic cells. This protein causes a four-fold stimulation of reverse transcriptase activity (Padhy *et al.*, 1976; Das *et al.*, 1978). We have also reported the presence of an inhibitory activity to reverse transcriptase in plasma membrane preparations from chicken embryonic cells (Das *et al.*, 1978). An examination of plasma membrane preparations from non-embryonic sources such as rat liver, a rat hepatoma (Zajdela ascitic hepatoma, ZAH) and a rat hepatic fibrosarcoma (Yoshida ascitic fibrosarcoma, YAS) has now shown that although no stimulatory factor is present in plasma membranes from these sources, there is a strong inhibitory activity in both normal and tumor plasma membranes. We report here the identification and characterization of this activity, and demonstrate that the inhibition is brought about by the binding of the inhibitor to the RNA template.

Materials and methods

Unlabelled deoxynucleoside triphosphates and synthetic templates were obtained from PL Biochemicals. (Me-^{3}H)-TTP (56 Ci/mmol) and (^{3}H)-dGTP (8 Ci/

Abbreviations used: ZAH, Zajdela ascitic hepatoma; YAS, Yoshida ascitic fibrosarcoma; AMV, avian myeloblastosis virus.

mmol) were obtained from Amersham-Searle, and calf thymus DNA and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Chemicals for enzyme assays were obtained from Patel Chest Institute, New Delhi. All other chemicals were of analytical grade. Purified avian myeloblastosis virus (AMV) reverse transcriptase was a gift from Dr J. W. Beard, Life Sciences Building, St. Petersburg, Florida, USA.

Tumor cells: Zajdela ascitic hepatoma (ZAH) and Yoshida ascitic fibrosarcoma (YAS) cells were maintained by serial transplantation of the tumor in Wistar rats. ZAH cells were obtained from Dr. F. Zajdela, Institut du Radium, Orsay, France and YAS cells were obtained from Cancer Research Institute, Parel, Bombay.

Plasma membrane preparation: Plasma membranes were prepared by three different methods as described elsewhere (Ray, 1970; Aaronson and Touster, 1972; Lesco *et al.*, 1973).

Isolation of AMV-RNA: AMV-RNA was isolated from purified AMV using phenol-cresol extraction as described earlier (Das and Mink, 1979).

Enzyme assays: Reverse transcriptase was assayed as described earlier (Parnaik and Das, 1981). 5'-Nucleotidase (Gurd and Evans, 1974), succinic dehydrogenase (Earl and Korner, 1965), alkaline phosphatase (Pekarthy *et al.*, 1972), alkaline phosphodiesterase I (Pekarthy *et al.*, 1972), inorganic pyrophosphatase (Shatton *et al.*, 1981) and glucose-6-phosphatase (Swason, 1955) were assayed as described.

Protein estimation: Protein concentrations were estimated by a modification of Lowry's method (Lowry *et al.*, 1951). Membrane suspensions (0.25 ml) were added to 1 ml alkaline tartarate solution (0.2% Na, K tartarate, 2% sodium carbonate and 0.002% cupric sulfate in 0.1M NaOH) and incubated at room temperature for 20 min. SDS (0.1 ml, 0.5%), and Folin's reagent (0.1 ml 2N) were then added in quick succession, and the mixture was vortexed. Absorbance at 740 nm was measured after keeping the samples at room temperature for 30 min. Bovine serum albumin solutions of known concentrations were used as standards.

Results

Inhibition of reverse transcriptase

Plasma membranes from normal rat liver, ZAH and YAS cells were isolated and assayed for purity by determining the enrichment of marker enzymes. Plasma membrane markers (5'-nucleotidase, alkaline phosphodiesterase I and alkaline phosphatase) were enriched 10-20 fold, whereas cytoplasmic contaminants (succinic dehydrogenase, inorganic pyrophosphatase and glucose-6-phosphatase) were undetectable. The membrane preparations were solubilized in 1% NP-40 and added to reverse transcriptase reaction mixtures. The final concentration of NP-40 was adjusted to 0.5% in the reaction mixtures. The concentration of membrane preparations ranged from 0.1 to 0.5 mg/ml according to the requirement of the experiments and are shown in the legends to tables and figures for individual experiments. Membrane preparations from normal liver as well as ZAH and YAS cells inhibited the copying of ploy rA.oligo dT by reverse transcriptase (figure 1). The drastic inhibition by liver plasma membranes was

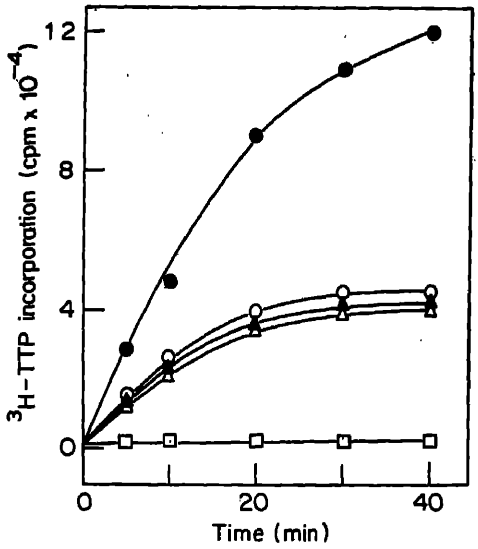

Figure 1. Reaction mixtures containing enzyme (0.72 U), 0.1 ml of solubilised plasma membranes in 1% NP-40 (30 µg of total protein) and components of the assay mixture (with poly rA.Oligo dT as template) in a total volume of 0.2 ml were incubated at 37°C. Aliquots (25 µl) were removed at various time intervals and assayed for reverse transcriptase activity as described in Methods. (●) Enzyme alone (in 1% NP-40); (▲) enzyme and liver plasma membrane; (△) enzyme and ZAH plasma membranes; (○) enzyme and YAS plasma membranes; (■) liver plasma membranes and enzyme in presence of 0.02 mM cysteine. ZAH plasma membranes and enzyme in presence of 0.02 mM cysteine showed the same kinetics as ZAH plasma membranes and enzyme in the absence of cysteine.

partly due to high levels of alkaline phosphatase activity in the plasma membrane preparations, as shown later. Due to this interfering inhibitory activity in liver plasma membrane preparations, most of the studies on the mechanism of inhibition were performed with tumor plasma membranes. As shown in figure 2, the inhibition was a linear function of the plasma membrane concentration, indicating that the inhibition was specific. The inhibitory activity was non-dialysable, suggesting that it was macromolecular in nature.

In order to determine whether the inhibitor was a protein, the plasma membrane preparations were treated with trypsin, followed by the addition of soybean trypsin inhibitor to inactivate trypsin, and then assayed for inhibition of reverse transcriptase. The inhibitory activity was destroyed by treatment with trypsin, indicating that the inhibitor is a protein (see table 1). Furthermore, inhibitory activity was abolished by incubating the solubilized membranes at 80°C for 5 min.

The possibility that the inhibition could also arise from the presence of any endogenous RNA or DNA in plasma membrane preparations that would competitively bind to the enzyme and inhibit poly rA.oligo dT copying was ruled out in the following manner. Membrane preparations were assayed for their ability to support DNA synthesis by reverse transcriptase by the addition of purified membranes (in 0.5% NP-40) to assay mixtures containing enzyme, all four dNTPs and oligo dT as primer but no other added template. Under these circumstances no

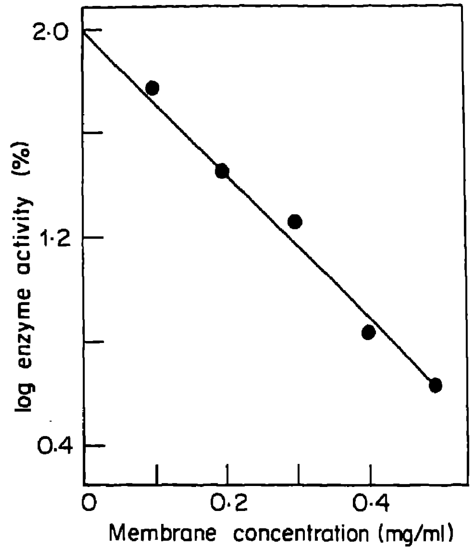

Figure 2. Enzyme samples (0.72 U) were treated with different concentrations of ZAH plasma membranes in 1% NP-40 (0.1 to 0.5 mg/ml) and assayed for activity (with poly rA.oligo dT as template) after 20 min at 37°C as described in Methods.

Table 1. Effect of trypsin treatment on the plasma membrane inhibitor.

Experiment	^{3}H-TTP incorporation (pmol/40 min./0.15 ml)
Standard reaction	318
Reaction with ZAH membranes	95
Reaction with trypsin-treated ZAH membranes	270
Reaction with added trypsin+soybean trypsin inhibitor (mixed prior to addition of enzyme)	305

ZAH plasma membranes (20 µg in 0.5% NP-40) were treated with 1 mg trypsin for 20 min. at 37°C, followed by the addition of 3 mg of soybean trypsin inhibitor. After 20 min. at 37°C, 1.0 U of reverse transcriptase and other components of the assay were added to give a total volume of 0.15 ml, and activity was determined as described in Methods. A control reaction with 0.5% NP-40 in the absence of plasma membranes was carried out in an identical manner. Values for control reactions in the absence of trypsin and SBTI treatment are also indicated. The specific activity of ^{3}H-TTP used was 500 cpm/pmol.

DNA synthesis was evident. The data strongly suggests that the inhibition is specifically brought about by a plasma membrane protein. The inhibitory activity was reproducibly observed from several batches of plasma membrane preparations, isolated by different methods and stored over varying lengths of time.

A series of experiments were carried out to test whether the observed inhibition could arise from the presence of any protease or nuclease activities in the membrane preparations. These were ruled out (results not shown). Protease activity was checked by the Kunitz method (Kunitz, 1947), using standard casein solutions. Ribonuclease and polynucleotide phosphorylase activities were assayed using *Escherichia coli* total RNA as substrate. Any interfering deoxyribonuclease activity in the membrane preparations was ruled out by the absence of degradation of DNA in incubation mixtures containing added DNA and membrane samples (results not shown).

As indicated earlier, liver plasma membranes contained considerable amounts of alkaline phosphatase, whereas tumor plasma membranes contained much lower amounts. In order to determine whether inhibition of reverse transcriptase by the membrane preparations was due to alkaline phosphatase activity, assays were carried out in the presence of 0.02 mM cysteine, a known inhibitor of alkaline phosphatase. Under these conditions, there was no effect on inhibition of reverse transcriptase by tumor membranes. However, the level of inhibition by liver plasma membranes decreased to the level of inhibition by tumor plasma membranes. It is clear from these experiments that whereas there was a genuine inhibition of reverse transcriptase by normal and tumor plasma membranes, the difference in the levels of inhibition by different membranes was due to differences in alkaline phosphatase levels in the membrane preparations. In order to check the presence of other phosphatases in membrane preparations, membranes were incubated with dNTPs. Under these conditions no degradation of dNTPs was observed.

We conclude from these experiments that the inhibition of reverse transcriptase by the membrane preparations is not caused by ribonuclease deoxyribonuclease, protease or phosphatase activities present in the membranes, but is caused by a specific protein inhibitor.

Mechanism of inihibition

The binding of the inhibitor either to the template or the enzyme could result in the observed decrease in DNA synthesis by reverse transcriptase in presence of plasma membrane preparations. If the inhibition is caused by the binding of the inhibitor to the enzyme, then at limiting concentrations of the inhibitor, increase in enzyme concentration should relieve inhibition. However, it was observed that as the enzyme concentration was increased five-fold, the percentage of inhibition did not vary significantly, indicating that the decrease in DNA synthesis was independent of enzyme concentration.

In order to determine whether the inhibitor acts by binding to the nucleic acid template, the effect of increasing template concentration on the extent of inhibition was studied. At limiting concentrations of the inhibitor, an increase in template concentration should relieve inhibition if the inhibitor acts by binding to the template. As shown in figure 3, inhibition is relieved considerably as the template concentration is increased. This observation strongly suggests that the inhibitor acts by binding specifically to the nucleic acid template.

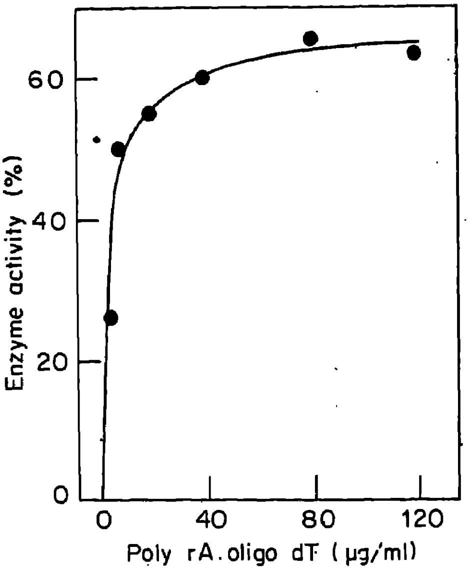

Figure 3. ZAH plasma membranes (10 µg) were added to reaction mixtures containing enzyme (0.24 U), various concentrations of poly rA.oligo dT (4-120 µg/ml) and the rest of the assay components in a volume of 0.1 ml. Enzyme activity was assayed after 10 min. at 37°C as described in Methods. Control reactions in the absence of membranes were carried out in an identical manner at each template concentration.

Template-specificity of inhibitor

The binding of the inhibitor to nucleic acid templates was critically examined using different templates. It can be seen from table 2 that the plasma membrane inhibitor specifically inhibits the copying of RNA templates including native AMV RNA, and has no detectable effect on the copying of two DNA templates: poly dA.oligo

Table 2. Template specificity of inhibitor.

Template	^{3}H-TTP incorporation (pmol/30 min/0.1 ml)		Inhibition %
	−Inhibitor	+Inhibitor	
Poly rA.oligo dT	339.0	97.9	73
Poly rC.oligo dG	3120.0	107.0	97
AMV RNA	9.8	3.0	69
Poly dA.oligo dT	8.0	8.0	0
Activated calf thymus DNA	20.8	19.3	7

Enzyme samples (0.1-2.0 U) were assayed with different templates (20 µg/ml) in the presence of 20 µg ZAH plasma membranes in a total volume of 0.1 ml. Enzyme activity was determined after 30 min. at 37°C as described in Methods. The specific activity of ^{3}H-dNTP (500-5000 cpm/pmol) was varied to give optimum incorporation of radio-activity for the different templates.

dT and activated calf thumus DNA. Furthermore, inhibition is considerably higher with the G, C-containing template, poly rC.oligo dG, suggesting that the inhibitor has a stronger binding affinity for G,C residues.

Experiments were also carried out to check whether the inhibitor specifically affects chain initiation. Rates of DNA synthesis were measured for two sets of parallel assays. In one set, reactions were started by the simultaneous addition of the enzyme and inhibitor to assay mixtures containing all the components of the reaction except the enzyme. In the second set, reactions were allowed to proceed for 5 min by adding aliquots of enzyme alone to the assay mixtures. At this point samples of membrane preparations were added to the reaction mixtures in this set. Both sets of reaction mixtures were incubated at 37°C and the incorporation of labelled TTP was monitored upto 40 min. The results showed no difference in the rates of incorporation of label between the two sets (data not shown). The absence of enhanced inhibition in reactions in which the inhibitor was present at start rules out the possibility of specific inhibition during the chain initiation event.

Discussion

A protein present in plasma membrane preparations from several sources such as normal liver cells, ZAH and YAS cells inhibits RNA-directed DNA synthesis by reverse transcriptase. Our results suggest that a template-inhibitor interaction is responsible for the observed inhibition. The inhibitor interacts specifically with RNA templates, and has a stronger affinity for G, C-containing polynucleotides. Preliminary experiments suggest that the inhibitor is present in membrane preparations from several other sources such as chicken liver cells and neonatal mouse liver cells. Recently, inhibitors of reverse transcriptase have been detected in mouse spleen cells (Rokutanda *et al.*, 1982) and human placental tissue (Nelson *et al.*, 1981). However, the cytoplasmic origin of both inhibitors makes it unlikely that they are similar to the inhibitor we have identified in plasma membranes.

The presence of an RNA-binding protein in plasma membranes from diverse sources raises the question of the functional role of such a molecule. Since replication of the retroviral genome proceeds via an RNA-dependent DNA polymerization, the inhibitory role of an RNA-binding protein could, in principle, provide a protective mechanism for the host. It has also been suggested that RNA-binding proteins might be involved in such diverse phenomena as the induction of interferons by double-stranded RNA molecules (Gordon and Hinks, 1981), modulation of host cell mRNA translation during infection by DNA tumor viruses (Khandjian *et al.*, 1982), compartmentalization of cytoplasmic RNA (DeRobertis *et al.*, 1982) and uptake of RNA by cells (Schell, 1971). It is not known whether these events take place at the cell surface or through the mediation of the plasma membranes. At present we do not know whether the RNA binding protein we have characterized in this study plays a role in any of these cellular events. Purification and further characterization of RNA-binding proteins from plasma membranes are under progress with a view to answer some of these questions.

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Biological characteristics of a factor suppressing follicle stimulating hormone (inhibin) from ovine testes

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Abstract. Inhibin (follicle stimulating hormone suppressing factor) isolated from ovine testes has been characterized for its biological activity using a variety of tests. The bioassay used—inhibition of the human chorionic gonadotropin induced increment in the mouse uterine weight—demonstrates that there is a significant increment in specific activity (approx. 300-fold) with the progress of purification. Eventhough the final product has not been obtained in a homogenous state it has been possible to show that (*a*) [^{125}I]-labelled inhibin is preferentially taken up and retained by the pituitary, pretreatment of rats with testosterone facilitating this uptake; (*b*) it is able to suppress specifically the levels of follicle stimulating hormone in castrated as well as immature intact rats and (*c*) treatment of immature male rats with inhibin preparation for ten days results in impairment of testicular function as judged by ^{3}H-thymidine incorporation into testicular DNA and testicular hyaluronidase activity.

Keywords. Regulation of FSH; testicular function; gonadotropin.

Introduction

Attempts are continuing to be made in several laboratories to isolate the follicle stimulating hormone (FSH) suppressing factor (inhibin) in a homogenous state from the gonad or its secretions. Although partial purification of inhibin from ovine testes has been reported earlier both by Murthy *et al.* (1979) and Sheth *et al.* (1979) the type of molecule each of these groups have isolated appear to be distinctly different. While, we have reported that the FSH release suppression activity is associated in the testis with a protein fraction whose molecular weight is around 15,000 daltons, Sheth *et al.* (1979) noted that FSH suppressing activity is exhibited by a low molecular weight peptide of approximately 1500 daltons, which they have isolated. From the ram rete testicular fluid, reports on the isolation of both types of material exist (Baker *et al.*, 1976). The low molecular weight material in addition to suppressing FSH secretion in castrated rats (Moodbidri *et al.*, 1980) appears to inhibit binding of [^{125}I]-FSH to rat testicular and ovarian membrane preparations (Vijayalakshmi *et al.*, 1980). It was therefore considered essential before continuing with the isolation procedure to understand better the biological

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Abbreviations used: FSH, Follicle stimulating hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone.

characteristics of the inhibin isolated by us from the ram testes. We wish to report here some of the results of our studies using the immature rat and mouse as animal models.

Materials and methods

Isolation of ovine testicular inhibin

Inhibin was isolated from ovine testes using conventional protein purification techniques, the details of which have been provided earlier (Murthy *et al.*, 1979).

The preparations P-1 to P-5 used here refer to the active fractions obtained by ammonium sulphate fractionation followed by chromatography on Sephadex G-75, DEAE, CM-cellulose and Sephadex G-50 respectively.

Bioassay of inhibin

Inhibin activity of the various fractions were determined using the mouse uterine weight assay described earlier by Ramasarma *et al.* (1979). This assay is based on the ability of inhibin, when given in two equal doses 1 h prior to human chorionic gonadotropin injection, to suppress the hCG-stimulated increase in uterine weight of 27 day old Swiss albino female mice. The usefulness and validity of this assay in quick screening of bioactivity of inhibin preparations has earlier been discussed (Ramasarma *et al.*, 1979).

Radioimmunoassay (RIA) of rat FSH and luteinizing hormone (LH)

These hormones were assayed using the rat FSH and LH RIA kits provided by NIAMDD, Bethesda, USA. The hormones were iodinated using carrier-free [^{125}I]-Na (Amersham, UK) by the chloramine-T method. The assay done according to Moudgal *et al.* (1979), consisted of incubating serum samples with the appropriate dilution of antiserum and the label at 37°C for 24 h, the antigen antibody complex being precipitated by the addition of goat antibody to rabbit gamma globulin and continuing incubation for an additional 12 h at 37°C. The coefficient of variation for intra and interassay were 3 and 9% respectively.

Radioiodination of inhibin and uptake studies with [^{125}I]-inhibin

The iodination of inhibin preparation (P-5) was carried out using the lactoperoxidase method (Moudgal *et al.*, 1979). The iodinated protein was separated from free iodine and lactoperoxidase by chromatography on Sephadex G-50 and hydroxylapatite column. Hydroxylapatite (column chromatography grade, BioRad, USA) packed in a 2 ml glass syringe was equilibrated with 0.005M phosphate buffer, pH 6.65, containing 0.1% bovine serum albumin (BSA). The absorbed inhibin was eluted with 0.1M phosphate buffer, pH 6.65 containing 0.1% BSA.

The [^{125}I]-labelled inhibin was injected to 23 day old immature male albino rats (I.I.Sc. strain), by *i.v.* route (2×10^6 cpm [^{125}I]-inhibin/200 µl of phosphate buffered saline) and groups of rats autopsied at periods thereafter. The different tissues obtained at autopsy were carefully blotted, weighed to the nearest 0.2 mg and 50-70 mg portions of these transferred to tubes and monitored for radioactivity in a Packard Autogamma spectrometer. In some cases, the tissue was homogenized with cold 10% TCA, and the radioactivity associated with the protein pellet determined.

Assessment of testicular function

Testicular function in immature male rats following chronic treatment (for 10 days starting from day 23) with inhibin was assessed using two biochemical parameters—*in vitro* ^{3}H-thymidine incorporation into testicular DNA and hyaluronidase activity—as indices of normal function. The methods used to determine the above are essentially similar to those described earlier for monkey testicular biopsy samples by Sheela Rani *et al.* (1978). For ^{3}H-thymidine incorporation studies, 30-50 mg of testicular tissue in triplicate was incubated in 1 ml of Krebs Ringer biocarbonate buffer, pH 7.4 containing 50 mM HEPES, 0.2% D-glucose and 1.0 µCi ^{3}H-thymidine (Radiochemical Center, Amersham, UK, specific activity 26 Ci/mmol). The tissue was incubated at 34°C for 4 h in a Dubnoff metabolic shaker. At the end of the incubation period 50 µg of non-radioactive thymidine (Sigma Chemical Co., USA) was added and the samples were processed for DNA by precipitating the macromolecules with 5% cold perchloric acid followed by hydrolysis with 10% perchloric acid at 90°C for 20 min. Aliquots of the filtrate were used for measurement of radioactivity in a Packard Tricarb Spectrometer as well as determination of DNA according to Burton's modified procedure (Giles and Myers, 1965). The results are expressed as cpm ^{3}H-thymidine incorporated/100 µg DNA per 4 h.

The hyaluronidase activity of the testes of rats was determined essentially according to the procedure of Males and Turkington (1970) as standardized in this laboratory (Sheela Rani *et al.*, 1978). The protein estimation was done according to the method of Lowry *et al.* (1951). The results are expressed as µmol of N-acetyl glucosamine (NAGA) released per mg protein per h.

All chemicals/biochemicals used in this study, unless otherwise mentioned, were of analytical grade and were purchased with Sigma Chemical Co., St. Louis, Missouri, USA or Sarabhai Chemical Co., India.

Statistical analysis

The results of this study were analyzed using the students '*t*' test.

Results

Characteristics of the inhibin preparation used

The final product (P-5) of purified inhibin on polyacrylamide disc gel electrophoresis (both at pH 8.2 and 4.5) showed one major and one/two minor bands (data not shown). The biactivity of the active fractions increased significantly with fractionation (figure 1). The amount needed to bring about 50% inhibition in uterine weight was reduced from an initial 7.7 mg (for P-1) to .019 mg of the P-5 fraction indicating that approximately 300-fold purification was achieved by this method.

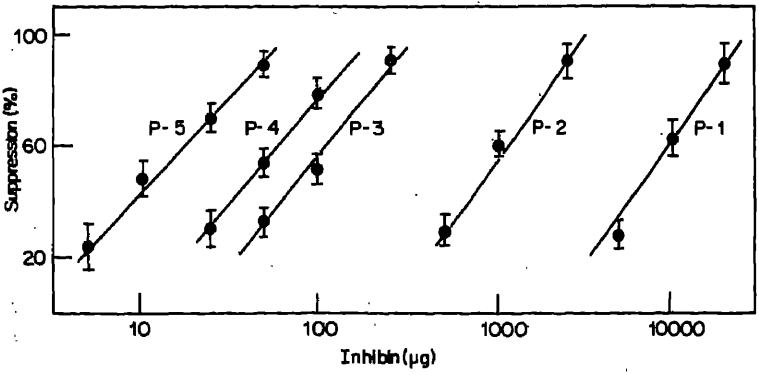

Figure 1. Dose response curves for inhibin activity as tested in the mouse uterine weight assay.

The different inhibin fractions (P1-P5) were tested at 3 dose levels and the results are expressed in terms of per cent suppression in uterine weight. This was calculated as follows:

$$100 \times \frac{(100\text{-uterine wt. of hCG}+\text{inhibin group}) - (\text{uterine wt. of control group})}{(\text{Uterine wt. of hCG group}) - (\text{uterine weight of control})}$$

Relative ability of different tissues of the rat to concentrate [^{125}I]-labelled inhibin

Groups of immature male rats, 23 days of age, were injected [^{125}I]-inhibin (P-5: 2×10^6 cpm/200 μl) *i.v.* and tissues obtained following sacrifice at ½, 1, 2, 4 and 8 h were analyzed for radioactivity. Of the various tissues analyzed, the pituitary appeared to retain maximal radioactivity for the longest period of time (figure 2).

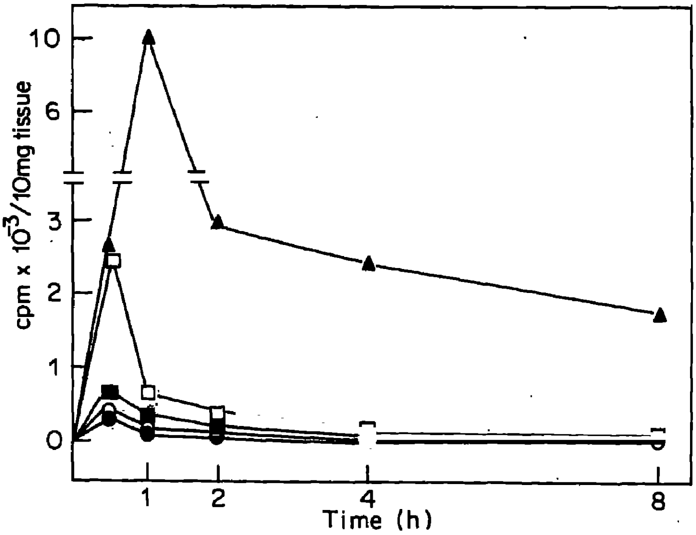

Figure 2. Distribution of [^{125}I]-inhibin into different tissues as a function of time, following its injection in immature rats.

23 day old immature male rats were injected 2×10^6 cpm [^{125}]-inhibin (P-5 by *i.v.* route). Animals were autopsied at various times thereafter and bound radioactivity (TCA precipitable fraction) in different tissues were monitored. *n* of each group — 4.

▲—Pituitary. □—Kidney. ■—Adrenal. ○—Liver and ●—Lung.

The radioactivity associated with the pituitary was precipitable with 10% TCA indicating that radiolabelled inhibin and not free iodine was being monitored. The ability of the pituitary to concentrate [^{125}I]-inhibin could be increased 3-fold by pretreating immature rats for 3 days with 1 mg testosterone per day (table 1).

Table 1. Effect of treating immature rats with testosterone on the uptake of [^{125}I]-labelled inhibin by the pituitary.

Treatment	cpm/10 mg tissue mean ± S.D.
Control	982±401
Testosterone	2917±914*

Testosterone propionate (1 mg/day) was injected s.c. to 23 day old immature intact male rats for 3 days in groundnut oil, controls being given vehicle only. 100,000 cpm of freshly labelled inhibin (P-5) in 200 µl of saline was injected *i.v.*, on day 26 of age and animals were sacrificed 1 h later. *n* of each group was 5.

* Significantly different from the control $P<0.01$.

Relative ability of testosterone and inhibin to suppress FSH levels of castrate rats

Within one week of castration, serum FSH levels of adult male rats were known to be significantly increased. While injection of 2 mg of testosterone per day for 3 days starting from day 7 of castration was unable to reduce the increased level of FSH of castrate rats, injection of 2 mg of inhibin preparation (P-3) for the same 3 day period did bring about a 20% reduction in serum FSH levels. The ability of inhibin to suppress serum FSH level, however, was further increased (by 43%) if administered along with testosterone (table 2).

Table 2. Ability of inhibin and testosterone to reduce serum FSH level in long term castrate rats.

Treatment	FSH* (µg/ml) mean±SD	Suppression (%)
Untreated controls	4.13±0.85	–
Testosterone	4.26±0.57	–
Inhibin	3.28±0.18	21
Testosterone + inhibin	2.35±0.09†	43

Adult male rats were castrated 7 days prior to start of therapy.

Inhibin (fraction P-3) and testosterone were injected, each at a dose 2 mg/day in two equal doses in the morning and evening for 3 days. Rats were sacrificed 16 h after the last injection. *n* of each group = 5.

* Expressed as rFSH-RPI NIH standard.

† Significantly different from the appropriate control $P<0.001$.

Effect of long term administration of inhibin on testicular function of immature rats

Immature male rats, 23 days of age, were administered s.c. inhibin (preparation P-3) at 1 mg/day for 10 days. The rats were autopsied 16 h after the last injection. The testis and accessory sex glands dissected and weighed to the nearest 0.2 mg showed no significant change in their weights (table 3). Analysis of serum for LH and FSH concentrations showed that inhibin treatment did bring about 33% reduction in FSH levels while LH levels remained unchanged (table 4). The two biochemical parameters used to assess testicular function—^{3}H-thymidine incorporation into testicular DNA and testicular hyaluronidase activity—showed significant reduction (by 25%) in their activities (table 5).

Table 3. Effect of long term administration of inhibin to immature male rats on the weight of testis and accessory sex glands.

Tissue	Weight of organs in mg (mean ±SD)	
	Control	Inhibin treated
Testis	802±39.9	721±23.1
Epididymis	88.9±6.89	103±8.65
Seminal vesicle	13.8±1.16	14.4±3.68
Prostate	33.0±3.71	40.2±5.25

Inhibin (fraction P-3, 1 mg/day) was administered to immature male rats for 10 days starting from day 23 of age. Each tissue was excised, blotted and weighed to the nearest of 0.2 mg. The rats were autopsied 16 h after the last injection. *n* of each group — 6.

Differences not significant by '*t*' test.

Table 4. Effect of long term administration of inhibition to immature male rats on serum FSH and LH.

Treatment	FSH (μg/ml) mean±SD	LH (ng/ml) mean±SD
Control	2.2±0.28	69± 4.1
Inhibin	1.4±0.16*	60±11.9
Per cent change over control	33	—

For treatment schedule and duration of treatment see footnote to table 3. The FSH and LH are expressed in terms of rFSH and rLH NIAMDD standards.

n of each group — 6.

* Significantly different from the control P — <0.01.

Table 5. Effect of long term administration of inhibin to immature male rats on testicular hyaluronidase activity and *in vitro* ^{3}H-thymidine incorporation into testicular DNA.

Treatment group	Hyaluronidase activity*	^{3}H-Thymidine incorp. (cpm × 10^4/100 μg DNA/4 h)
Control	610±74.5	23.7±2.3
Inhibin	449±27.6**	17.5±0.6**
Per cent change over control	26	25

See foot note of table 3 for details of experimentation.

n of each group = 6.

The hyaluronidase activity was determined in triplicates using one testis of each animal. The other testis from individual animals of a group were pooled and the *in vitro* ^{3}H-thymidine incorporation studies into testicular DNA were carried out using triplicate samples.

* Expressed as μmol of N-acetyl glucosamine (NAGA) released per mg protein per h.

** Significantly different from control ($P<0.001$).

Discussion

An attempt has been made here to study the biological effects of the FSH suppressing factor (inhibin) isolated in this laboratory from the ovine testes. The inhibin preparations as tested in immature female mice are able to inhibit the hCG-induced increment in uterine weight in a dose-dependent manner and the specific activity, as to be expected, increased with the progress of fractionation. The validity of using the mouse bioassay for measuring FSH suppressing activity has earlier been provided. Although the method of purification adopted here has not provided an electrophoretically homogenous product (P-5 shows the presence of one major and 1-2 minor bands in disc gel electrophoresis), it was still felt desirable to biologically characterize the product using different systems which could monitor inhibin activity before improving on the fractionation procedure.

One of the recommended procedures for assaying inhibin activity is the pituitary cell culture method (Hudson *et al.*, 1979). This as well as other studies (de Jong *et al.*, 1979; Scott *et al.*, 1980) suggest that the pituitary could be the principle target organ of inhibin action and Sairam *et al.* (1981) have also described a receptor assay for inhibin using crude pituitary homogenates. The present studies with [^{125}I]-labelled inhibin essentially show that the pituitary, relative to other organs monitored, preferentially retains labelled inhibin and of particular interest is the observation that pre-treatment of rats with testosterone facilitates this ability of the pituitary to retain the labelled compound. This essentially supports the suggestion that the pituitary has receptors to inhibin. Vanage *et al.* (1980) have reported using [^{125}I]-labelled inhibin preparation isolated from human seminal plasma, that it is preferentially taken up in addition to the pituitary, by the pineal of rats. The uptake appears to be a function of the age of the animal, maximal uptake being exhibited by 18 day old male rats. Recently, by implanting highly purified inhibin preparation in the third ventricle of unanaesthatized intact and castrated rats, a specific reduction in plasma FSH levels has been observed, suggesting thereby that the hypothalamus could also be one of the sites of action of inhibin (Lumpkin *et al.*, 1981; Nagesh Babu *et al.*, 1981).

The castrate rat model used here permits one to assess the intrinsic ability of inhibin to suppress serum FSH levels. Though testosterone by itself is unable to suppress the FSH levels of long term castrate rats, essentially confirming the observation of Morger (1976) and Juneja *et al.* (1976), it appears to potentiate the ability of inhibin to do so. The mechanism whereby this is effected is presently unknown. Our earlier studies in the long term castrate monkey had similarly shown that inhibin has intrinsic ability to suppress FSH levels (Murthy *et al.*, 1980).

It is now known that in immature rats, neutralization of serum FSH with a specific FSH antibody results in significant inhibition of the spermatogenic process (Raj and Dym, 1976; Shivashankar *et al.*, 1977); the adult male rat, however, does not appear to be dependent upon FSH to support spermatogenesis (Lostroh, 1976). Administration of inhibin to immature male rats for a ten day period starting from day 23 of age also seems to result in inhibition of testicular function as adjudged by its effect on ^{3}H-thymidine incorporation into testicular DNA and testicular hyaluronidase activity, used here as indices of active spermatogenesis and tubular functionality respectively. These parameters have been shown earlier by us to be effected following FSH antiserum treatment of adult male monkey (Sheela Rani *et al.*, 1978) as well as of immature male rats (Dhanasekaran and Moudgal, unpublished observations).

Although the reduction observed in both these parameters is of the order of only 25%, on the basis of specific activity (35% if expressed per pair of testes) it was statistically significant ($P<0.001$). It is possible that instead of using whole testicular tissue as in the present case, if enriched tubular preparation had been used the reduction observed may have been more marked. Franchimont (1982) interestingly, has also observed that following injection of an inhibin preparation isolated from rete testicular fluid of sheep to immature rats, a reduction in ^{3}H-thymidine incorporation into testicular DNA as well as uptake by type B spermatogonia occurs. Using bovine follicular fluid treated with charcoal as a source of FSH suppressing factor, in immature male rats, de Jong *et al.* (1978) observed a retardation in spermatogenesis and in particular, decrease in the number of pachytene spermatocytes. In the monkey, an inhibition in ^{3}H-thymidine incorporation into testicular DNA and testicular hyaluronidase activity have been correlated with a marked reduction in total number of sperms voided and a loss in fertility (Murthy *et al.*, 1980). Although, inhibin injection to immature rats resulted only in 33% reduction in serum FSH levels (in contrast to total neutralization of FSH achieved by giving antiserum), this appears to be adequate to effect testicular function showing that there is a critical need for FSH to support spermatogenesis during the immature state. The specificity of the effect of inhibin on FSH-dependent function is evident from the fact that the weights of the accessory sex glands, a sensitive index of testosterone (and as such LH) availability did not show any change.

It is thus possible to conclude from the present study that the procedure used by us to isolate inhibin does provide a product which exhibits properties expected of a factor which specifically suppresses FSH secretion. Preliminary data indicates that this factor does not possess FSH binding inhibiting activity (Bhat and Moudgal, unpublished observations).

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Ontogeny of insulin-receptor interaction: correlation with circulatory insulin levels*

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Abstract. Interaction of $[^{125}I]$-insulin with intact hepatocytes and its correlation with circulatory insulin level was examined. The hepatocytes from new-born rats bound lowest amount of $[^{125}I]$-insulin (1.39 ± 0.41 pM/mg cell protein) when circulatory insulin level was high (8 ± 1.5 μU/ml). Hepatocytes from 7 day and 21 day old animals demonstrated a more or less similar relationship. Cells from 31 day old animals exhibited maximum insulin binding activity (5.13 ± 0.18 pM/mg cell protein) against a low serum insulin level (4.25 ± 0.25 μU/ml). Scatchard analysis of insulin binding shows that the affinity is higher in the hepatocytes from new-born animals than in the hepatocytes of 31 day old animals. Higher binding observed in the latter case may be due to a greater number of binding sites. Hepatocytes from one year old rats bound very little insulin (2.50 ± 0.36 pM/mg cell protein) against a high circulatory insulin level (9.25 ± 0.85 μU/ml). In view of these results, it appears that the down-regulation hypothesis holds true during ontogeny too.

Keywords. Ontogeny; insulin receptor; serum insulin; hepatocytes.

Introduction

Insulin plays a key role in the homeostasis of circulatory glucose (Lehninger, 1978). Physiological action of insulin on the target tissues owes to its interaction with the specific membrane receptors (Heise *et al.*, 1982). Recent years have witnessed accumulation of sizeable information on insulin receptor interaction. However, our knowledge regarding the regulation of insulin receptors and their bioavailability is scant (Caro and Amatruda, 1980). Circulatory insulin is believed to influence bioavailability of its own receptors on the target tissues (Bar *et al.*, 1976; Davidson and Kaplan, 1977). It was considered of interest therefore, to investigate the ontogenesis of insulin-receptor interaction and find correlation, if any, with the circulatory insulin level. The present communication describes the same using intact rat liver-cells as a model.

Materials and methods

Chemicals and animals

Collagenase, Type V, porcine insulin (23. 6 I.U./mg) and bovine serum albumin (BSA) were the products of Sigma Chemical Co., St. Louis, Missouri, USA. $[^{125}I]$-

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* CDRI Communication No. 3074.

Abbreviations used: BSA, Bovine serum albumin; L-15, lebowitz-15; PBS, phosphate buffer saline.

Insulin (sp. activity 100 μCi/μg) was purchased from Bhabha Atomic Research Centre, Bombay. HEPES (N-2-hydroxyethyl piperazine N′ethanesulfonic acid) was from Sisco Research Laboratories, Bombay. Lebowitz-15 (L-15) medium was the product of GIBCO, USA. Albino rats of Charles Foster strain were used in the present study.

Preparation of hepatocytes

Hepatocytes from 21 day, 31 day and one year old animals were prepared by collagenase digestion according to Seglen (1976) and Berry and Friend (1969), using six animals in each case. In the case of new-born and 7 day old rats, the cells were obtained by direct incubation of minced liver with collagenase in phosphate buffer saline (PBS) containing 1 mM $CaCl_2$ supplemented with 25 mM HEPES (pH 7.4), 0.1% BSA and 0.1% glucose at 37°C. The number of animals used for the isolation of hepatocytes for new-born and 7 day old group was thirty each. The cells obtained were washed four times by suspending in PBS and centrifugation at 100 g for 2 min. Subsequently the hepatocytes were suspended in L-15 medium supplemented with 7 mM glucose, 25 mM HEPES, pH 7.4 for 30 min before commencement of the experiment. Viability of the hepatocytes was monitored by following exclusion of 0.05% trypan blue and it was found to be 90-95%.

Receptor assay

This was carried out according to procedure described elsewhere (Balapure *et al.*, 1980). Suitable aliquots of hepatocytes from rats of different ages were incubated with $1\text{-}3 \times 10^4$ cpm of [^{125}I]-insulin (sp. act. 100 μCi/μg), purified on Sephadex G-50 column, in PBS containing 1% BSA (buffer A). The incubation was carried out at 24°C for 90 min in a total volume of 1 ml with constant shaking. Native insulin in the concentration of 10^{-6}M was added to determine non-specific binding. After the incubation was over, the tubes were centrifuged at 100 g at 4°C for 4 min to separate the cell pellet and the supernatant. The cell pellet was washed again with 3 ml of chilled buffer A and centrifuged as before. The supernatants were pooled. Bound and free radioactivity was counted in a Packard Autogamma Scintillation Spectrometer Model 5230.

Radioimmunoassay of circulatory serum insulin

Blood was drawn from rats of different age groups by decapitation and allowed to clot. Serum was collected to estimate insulin levels by radioimmunoassay using double antibody procedure essentially according to Morgan and Lazarow (1963).

Protein estimation

Protein was estimated in the cell pellet according to Lowry *et al.* (1951) using BSA as standard.

Results

Ontogenesis of circulatory serum insulin levels

Figure 1 summarises the results obtained. A high serum insulin level was observed in new-born rats which declined progressively with age during the first three weeks of post-natal life. The insulin level again rose in 31 day old rats and the highest value was observed in a year old rats.

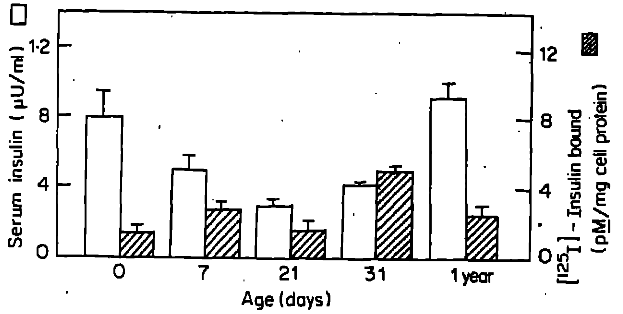

Figure 1. Radioimmunoassay of serum insulin □ and $[^{125}I]$-insulin binding to the hepatocyte insulin receptor ▨. Insulin level and $[^{125}I]$-insulin binding to the receptor was monitored according to Morgan and Lazarow (1963) and Balapure *et al.* (1980) respectively. Each point represents the Mean ±S.D. of six observations.

$[^{125}I]$-Insulin and hepatocyte insulin receptor interaction

Insulin-receptor interaction was detectable in hepatocytes from new-born rats as shown in figure 1. The same showed a small increase in the hepatocytes from 7 day old ones with a subsequent decline in 21 day old animals. Insulin binding activity again increased with a peak in 31 day old rats. Hepatocytes from one year old rats showed low insulin binding activity, yet it was higher than that observed in those from new-born rats.

Scatchard analysis (Scatchard, 1949) of $[^{125}I]$-insulin binding was evaluated in hepatocytes from new-born and 31 day old rats. It was observed that association constant (K_a) for high and low affinity $[^{125}I]$-insulin binding sites were $10.4 \times 10^9 M^{-1}$ and $5 \times 10^9 M^{-1}$ respectively for the new-born rat hepatocytes (figure 2 inset). The corresponding values for the hepatocytes from 31 day old animals were $4.64 \times 10^9 M^{-1}$ and $0.86 \times 10^9 M^{-1}$ respectively (figure 2).

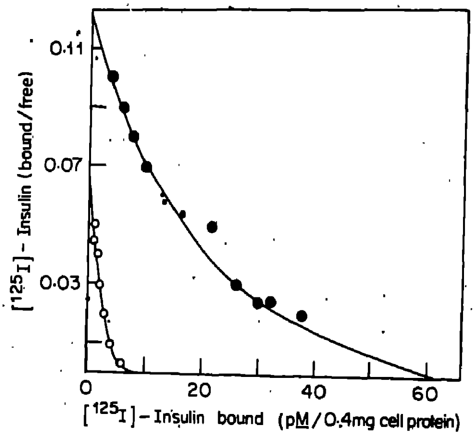

Figure 2. Scatchard plot of $[^{125}I]$-insulin binding to the hepatocyte insulin receptor from new born (inset, 1) and 31 day old (●) rats. Each point represents the average of three determinations.

Discussion

Ontogeny of insulin receptor from rat hepatocyte revealed an interesting pattern with low insulin binding in hepatocytes from new-born rats, rising to highest level in the hepatocytes from 31 day old rats to decline further with age to a low level in the cells derived from one year old rats. In contrast, the developmental profile of serum insulin level was almost inversely related to the pattern observed for the ontogeny of hepatocyte insulin receptor. High circulatory insulin level was observed in the serum from 31 day old rodents which increased further with age. It would appear that insulin in circulation and insulin receptors on hepatocytes bear a reciprocal relationship. Such a relationship between insulin and insulin receptors has been observed and defined as "down-regulation" (Gavin III *et al.*, 1974) under different *in vitro* as well as *in vivo* experimental conditions (Livingston *et al.*, 1978; Blackard *et al.*, 1978), barring a few exceptions (Amatruda *et al.*, 1975; Broer *et al.*, 1977; Livingston *et al.*, 1972; Misbin *et al.*, 1979; Smith and Digirolamo, 1980). That such a pattern is seen during development also is interesting and supports the view that a down regulation phenomenon could be operating during ontogeny as well.

Scatchard analysis of [^{125}I]-insulin-receptor binding was carried out to probe the reasons for the observed differences in insulin binding activity of hepatocytes from new-born (1.4 pM/10^6 cells) and 31 day old rats (5.1 pM/10^6 cells). It was observed that the association constants (K_a), both for the high and low affinity sites were higher for the hepatocytes from new-born rats. It would, therefore, appear that the higher insulin binding observed in the hepatocytes from 31 day old rats was due to an increase in the number of insulin receptors as opposed to enhanced affinity for insulin. This observation again supports the proposition that circulatory insulin level autoregulates the number of insulin receptors. It is not clear at the moment how insulin is able to regulate the bioavailability of insulin receptors. It has been suggested that insulin receptor interaction alone does not suffice to "down-regulate" insulin receptors (Amatruda *et al.*, 1975). Studies are in progress to evaluate the turnover of insulin receptors and its regulation by circulatory insulin. This will help in elucidating the mechanism of their bioavailability in normal and diseased conditions.

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Surface membrane changes in lepromatous macrophages affecting the adherence of *Mycobacterium leprae*

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Abstract. Macrophages from lepromatous leprosy patients showed poor adherence to *Mycobacterium leprae.* The phagocytic activity of the macrophages was not correlated to the influence on the adherence ability. Based on the phagocytic behaviour of macrophages from normal individuals and from lepromatous leprosy patients as well as the action of neuraminidase in reversing the extent of adherence, it is suggested that macrophages from lepromatous leprosy patients differ from those from normal individuals in regard to their surface properties. There was no relationship between the degree of adherence and the concentration of Fc receptors of the macrophages. It was also shown that an extract of lysed macrophages from lepromatous leprosy patient was able to reduce the adherence of *Mycobacterium leprae* to normal macrophages. This study shows that adherence is a good indicator of the surface property of macrophages which in turn could play an important role in the cell mediated immunity of the patient. The observations suggest altered macrophage membrane structure in the long term-treated, otherwise normal, lepromatous leprosy patients.

Keywords. *Mycobacterium leprae;* adherence; macrophage membrane; lepromatous leprosy.

Introduction

In an earlier report (Lad and Mahadevan, 1982), we had shown that *Mycobacterium leprae* adhered to human macrophages and that this adherence was based on chemical interaction. The ability of adherence of *M. leprae* to macrophages from lepromatous leprosy patient was significantly reduced and this defect was specific to *M. leprae.* One of the changes induced in the macrophages of lepromatous leprosy patients, was the alteration in the membrane structure indicated by changes in the levels of various receptors (to be published elsewhere). Furthermore, an extract from lysed macrophages of lepromatous leprosy patients, induced changes in the membranes of macrophages from normal individuals (Salgame *et al.*, unpublished observations). Studies on factors such as the phagocytic ability, the effect of soluble factors affecting adherence, role of Fc receptors and the innate membrane alterations that could be identified using

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Abbreviations used: Fc, Receptor for Fc region of immunoglobulins; BI, bacteriological index; T, tuberculoid type of leprosy patient; MEM, minimal essential medium; N- and L-lysate, lysate from macrophages from bacteriologically positive lepramatous patients and from macrophages from normal subjects to *M. leprae in vitro* respectively; SRBC, sensitized sheep red blood cells.

'adherence ability' as an indicator which influence the property of adherence of *M. leprae* to the macrophages from lepromatous patients and the relationship of this property to the membrane structure are reported in this communication.

Materials and methods

Normal subjects

These were healthy volunteers residing in a leprosy endemic area having varying degrees of exposure to leprosy bacilli.

Patients

Leprosy patients used in this study were classified according to the Ridley and Jopling (1966) classification.

Lepromatous leprosy bacteriologically-positive patients

These were lepromatous leprosy patients who were bacillary positive but receiving treatment, duration of which varied from a few months to several years. The degree of positivity was indicated by bacteriological index (BI) from skin smears when necessary (Ridley, 1964). The long-term treated, bacteriologically negative lepromatous leprosy patients are referred to as lepromatous negative. Tuberculoid type of leprosy patients are referred to as T.

Culturing of macrophages

Macrophages were cultured from peripheral blood as described by Lad and Mahadevan (1982). Briefly, packed leukocytes were obtained from peripheral blood. It was washed with minimal essential medium (MEM), (Centron Laboratories, Bombay) and dispensed in Leighton tubes after suspending in the culture medium (40% human AB serum in MEM with 100 units/ml of penicillin). On the 5th day, almost pure, glass-adherent cells, typical of macrophages, were obtained. The macrophages obtained from the peripheral blood of the leprosy patients are referred to as lepromatous macrophages or tuberculoid macrophages and those from normal individual as normal macrophages. *M. leprae* from human sources were obtained from freshly collected nodules from untreated lepromatous patients (Acworth Leprosy Hospital, Bombay) while *M. leprae* from Armadillo spleen (preserved continuously at −90°C) were obtained from tissues supplied by Dr E. Storrs (Melbourne, Florida, USA). The bacilli were harvested as described earlier (Lad and Mahadevan, 1982).

Adherence of bacilli to macrophages

The adherence of bacilli was carried out as described earlier (Lad and Mahadevan, 1982). Briefly, five day old macrophage cultures in Leighton tubes were exposed to 20×10^6 bacilli at 8°C for 1 h. The cells were washed, fixed with 2.5% glutaraldehyde and stained by the Zeihl-Neelson technique to identify the acid-fast bacteria adherent to macrophage. The enumeration of macrophages with adherent bacilli was carried out as described earlier (Lad and Mahadevan, 1982)

Phagocytosis

One set of macrophage cultures were exposed to *M. leprae* (5×10^6 bacilli per tube) directly at 37°C for 1 h. The second set of cultures were exposed to the bacilli at 8°C for 1 h and then incubated at 37°C for an additional 1 h. At the end of the incubation period, the cells, were washed, fixed and stained for acid-fast bacilli. One hundred macrophages were counted and those having phagocytosed bacilli were expressed as a percentage of the total.

Colchicine treatment of macrophages

Five-day old macrophages cultures from positive lepromatous leprosy patients were exposed to 10^{-5}M colchicine solution for 1 h at 37°C. At the end of the incubation period the cells were washed twice with saline and were then exposed to *M. leprae* to assess their ability to adhere.

Preparation of lysate

Lysate was prepared from macrophages of bacteriologically positive lepromatous patients (L-lysate) as described by Salgame *et al.* (1980). Lysate was also prepared from normal macrophages exposed to *M. leprae in vitro* (N-lysate). This is to simulate as closely as possible conditions prevailing in the lepromatous macrophage.

Treatment of macrophage with lysate

Five day old macrophage cultures were exposed to the above lysate (derived from 10^5 macrophages) at 8°C for 1 h. At the end of the incubation period, the cells were washed once with saline and used to determine the level of adherence to *M. leprae* (Lad and Mahadevan, 1982).

Evaluation of Fc receptors in relation to adherence

Adherence of *M. leprae* was carried out with macrophages pretreated with uninactivated human AB serum at 37°C for 1 h. Untreated macrophages served as control.

The blocking effect of Fc receptors by the antibodies in the serum was confirmed by using sensitised sheep red blood cells (SRBC). SRBC were sensitised by treating them with 1:3000 dilution of amboceptor (obtained from Haffkine Institute, Bombay), at 37°C for 1 h. At the end of the incubation period SRBC were washed thrice with saline and suspended to make a final concentration of 2% (v/v). The cells were assayed for the presence of Fc receptor as described by Birdi *et al.* (1980). Macrophages were exposed to sensitised SRBC at 8°C for 1 h to simulate the condition existing at the time of carrying out the adherence test.

Neuraminidase treatment of macrophages

Five day old macrophage cultures were exposed to neuraminidase (5 units/ml). (Sigma Chemical Co., St. Louis, Missouri, USA) at 37°C for 12 min according to the method described by Wier and Ogmudsdottir (1977). At the end of the incubation period, the cells were washed with saline and the adherence was determined as described above.

Results

Relationship of phagocytosis to adherence

Adherence does not appear to be related to the level of phagocytosis by the macrophages. This conclusion was based on the following data.

1) The level of adherence was very low in macrophages from bacillary-positive lepromatous patients and high in macrophages from bacillary-negative patients. Nevertheless the level of phagocytosis in the two types of macrophages was almost the same under both the experimental conditions. During direct incubation at 37°C for 1 h, the mean level of phagocytosis was close to 3% in macrophages of lepromatous leprosy-positive patients and near to 8% in macrophages of lepromatous leprosy-negative patients. During the tempeature shift from 8°C to 37°C it was 20 and 23% respectively (table 1).

Table 1. Level of phagocytosis of *M. leprae* by macrophages.

Source of macrophages	Per cent phagocytosis (Mean ± S.D.)	Incubation at 37°C/1 h
	Pre incubation at 8°C	Not preincubated
Normal	8 ± 1.7	33 ± 9.2
LL (+)	20.30 ± 4.0	2.67 ± 2.1
LL (−)	23.00 ± 12.0	7.7 ± 8.3
TT (III)	5.30 ± 2.5	11.30 ± 1.5

2) The level of phagocytosis in normal individuals is higher than in treated tuberculoid patients when incubated at 37°C for 1 h (normal — 33%, tuberculoid — 11%).

Normally, macrophages do not phagocytose ingestible materials, when incubated at low temperatures (24°C) (Pesanti and Nugent, 1981). This was clearly seen by the failure of normal macrophage to phagocytose armadillo-grown *M. leprae* when they were incubated in the cold (8°C) and then shifted to 37°C (8% phagocytosis).

3) Lepromatous macrophages, both bacillary-positive and bacillary-negative, do not behave like normal macrophages. Incubation at 8°C enhanced their phagocytic capacity unlike that of the normal macrophages (table 1) indicating differential phagocytic ability of the macrophages.

The level of adherence of *M. leprae* to normal macrophages and treated tuberculoid patients macrophages have been determined earlier as 14.33% ± 5.85 and 14.17% ± 5.42 respectively (Lad and Mahadevan, 1982).

Effect of lysate on adherence

The lysate derived from lepromatous leprosy bacillary-positive macrophages was able to reduce the level of adherence of the normal macrophages from 14% to 3%. On the other hand, lysate derived from normal macrophages failed to inhibit adherence ability of macrophages from normal individuals (figure 1).

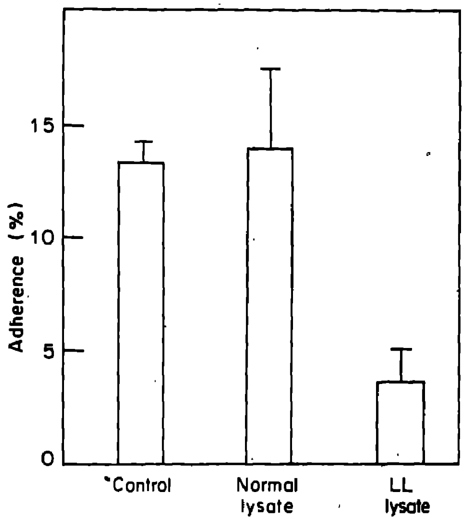

Figure 1. Effect of lysate on adherence of *M. Leprae* to normal macrophages.

The bar chart represents the mean of three experiments and standard error of mean.

Fc receptors are different from the receptors for M. leprae adherence

Pretreatment of macrophages with antibody inhibited the Fc receptor expression of macrophages to antibody coated SRBC, but failed to inhibit the ability of *M. leprae* to adhere to macrophages (table 2) indicating that Fc receptors may not be involved in adherence of the bacteria.

Effect of colchicine on adherence

The receptors present on the macrophages from normal individuals and treated bacillary-negative lepromatous patients were sensitive to colchicine treatment (figure 2), since it reduced the adherence ability.

In bacillary-positive lepromatous patients, colchicine treatment restored the level of adherence to near normal values (figure 2). Similar treatment failed to bring back the level of adherence in the macrophages of lepromatous patients who were only slightly positive (BI 1+).

Effect of neuraminidase on adherence

Neuraminidase treatment increased the level of adherence of *M. leprae* to macrophages from normal individuals (table 3). In the case of the macrophages from bacillary-positive lepromatous patients, there was also an increase in the level of adherence. It is interesting to note that neuraminidase failed to increase

Table 2. Effect of neuraminidase on adherence ability of *M. leprae* to macrophages.

Type of macrophages	Expt. no	Control cells		Neuraminidase treated cells	
		Per cent adherence*	Number of bacilli bound to 100 cells	Per cent adherence	Number of bacilli bound to 100 cells
Normal	1	14	n.d.	31	n.d.
	2	35	143	52	250
	3	15	55	26	155
LL (−ve)	1	35	114	26	55
	2	18	82	14	46
	3	14	44	13	38
LL (+ve)	1	2	14	22	76
	2	2	n.d.	15	n.d.
	3	2	9	25	94

n.d.—Not done.

* Number of macrophages adhering *M. leprae*.

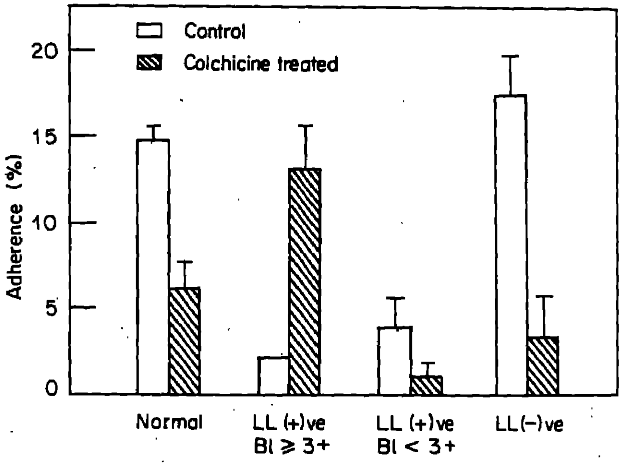

Figure 2. Effect of colchicine on adherence of *M. leprae* to macrophages.

The bar chart represents the mean of three experiments and standard error of mean.

adherence of *M. leprae* to macrophages from bacillary-negative patients. These observations clearly indicate that the lepromatous macrophages, (from bacillary negative patient) though they exhibit the same degree of receptor-mediated adherence, possibly the receptors being chemically identical to those of normal macrophages, are perhaps not exactly the same as normal macrophages in all respects.

Table 3. Fc receptor in relation to adherence of *M. leprae* to macrophages.

	Expt. no. 1	2	3
	Per cent adherence		
Control macrophages	14	35	16
Antibody-treated macrophages	27	29	40
	Per cent macrophages with Fc receptors[a]		
Control macrophages	46	25	35
Antibody-treated macrophages	10	3	00

[a] Per cent macrophages having two or more sensitised SRBC attached.

Discussion

Phagocytosis is an important property of macrophages and since the same macrophages also exhibit adherence to pathological organisms like *M. leprae*, it was essential to understand the relationship between these two phenomena. The observation that adherence ability of the macrophage of patients suffering from lepromatous leprosy has greatly reduced is of considerable significance. Our observations indicate that receptors involved in adherence do not seem to regulate the level of phagocytosis by the macrophages. Further more, the phenomenon of phagocytosis of macrophages from lepromatous patients is quite distinct from those of the normal. At 37°C normal macrophages showed good phagocytic ability which was absent when incubated at 8°C and remained so even after shifting the culture to 37°C after the prior cold incubation. At 37°C macrophages from the lepromatous-positive and negative patients showed low phagocytosis. The decreased level in this case could be due to a different membrane disposition that does not help phagocytosis. However when incubated at 8°C and then shifted to 37°C unlike the normal macrophages, those from both types of patients showed higher phagocytosis. This perhaps indicates that cold temperature brings about some favourable disposition in the membrane that enables increased phagocytosis at 37°C. This also indicates a possible innate difference between the two types of macrophages, the normal and that of the patient.

It has also been shown by the present study that the ability of adherence of normal macrophages is greatly reduced when such macrophages are exposed to the lysate of macrophage derived from lepromatous patients. The changes brought about by the lysate on the membrane may be responsible for the reduced adherence of bacteria similar to the effect with other receptors (Salgame *et al.*, 1980; unpublished observations). It could be possible that such products produced inside the macrophages make the cells exhibit poor adherence to *M. leprae* in the patient.

Our earlier results showed that the reduced level of adherence was reversed treatment of the macrophages with trypsin (Lad and Mahadevan, 1982). In t present work, it has been shown that colchicine can also reverse it. This reversal colchicine indicates the role of the cytoskeletal elements of the macrophages li the microtubules in the membrane alteration induced by *M. leprae*.

Fc receptors of macrophages do not play a role in adherence, since antibo treatment of macrophages failed to alter the ability of *M. leprae* to adhere macrophages. Hence the reduced adherence in normal macrophages treated wi the lysate is a clear indication that it is due to modification of receptors f adherence and not through alteration of receptors for Fc or phagocytosis.

Our data also demonstrate that neuraminidase treatment increased the level adherence of *M. leprae* to macrophages from normal individuals. On the oth hand, the long term-treated bacillary-negative lepromatous patients, who macrophages otherwise behave normally, are not altered by neuraminida treatment as regards their adherence ability.

These observations, like the different phagocytic ability of lepromato macrophages from that of the normal, and that neuraminidase treatment of the two types of macrophages elicit different responses, indicated to us that t macrophage of patients who are susceptible to, but not suffering from lepromato disease, could have an altered membrane structure. This alteration may be due to preexisting state in the individual which could be due to a gene function genetic otherwise. Our future studies are being directed towards clarification of the abo possible concept.

In conclusion, it can be stated that the study of the phenomenon of adherence *M. leprae* to macrophages, reveals some basic differences between th macrophages of the lepromatous leprosy patients and the normal. This may hav relevance to the cell mediated immunity of the host.

Acknowledgements

The authors wish to acknowledge the help from the Acworth Leprosy Hospital i supplying the human materials and Dr E. Storrs, Melbourne, Florida, USA for th supply of armadillo-grown *M. leprae*, which was aided by a grant from LEPRA UK.

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Plasma gonadotropin, prolactin levels and hypothalamic tyrosine hydroxylase activity in rats during estrous cycle, after overiectomy and after blockade of catecholamine biosynthesis

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Abstract. Plasma gonadotropin, prolactin levels and hypothalamic tyrosine hydroxylase activity were evaluated at 0900, 1200 and 1700 h during diestrus, proestrus and estrus, ovariectomized and after systemic administration of reserpine or α-methyl-*p*-tyrosine, which interfere with catecholamine biosynthesis, in rats. Gonadotropin and prolactin levels showed peak values during the afternoon of proestrus, while hypothalamic tyrosine hydroxylase activity was markedly lowered at 1200 on proestrus. Gonadotropin levels were slightly lowered whereas prolactin concentrations and hypothalamic tyrosine hydroxylase activity were significantly increased by reserpine. Depletion of hypothalamic dopamine by reserpine apparently resulted in significant elevation of prolactin levels which inturn induce tyrosine hydroxylase. Gonadotropin levels and hypothalamic tyrosine hydroxylase activity were significantly suppressed after the administration of α-methyl-*p*-tyrosine. Prolactin levels, however, were elevated significantly. These results indicate that catecholamines are involved in the control of gonadotropin and prolactin release during estrous cycle and inhibition of catecholamines biosynthesis by α-methyl-*p*-tyrosine could result in suppression of gonadotropin levels, whereas removal of tonic inhibition of hypothalamic dopamine by α-methyl-*p*-tyrosine elevate prolactin levels.

Keywords. Estrous cycle; ovariectomy; reserpine, α-methyl-*p*-tyrosine; gonadotropins; prolactin; tyrosine hydroxylase.

Introduction

Catecholamine (CA) containing neuronal systems terminating in the hypothalamus and other areas of central nervous system regulate or modulate the release of gonadotropins and PRL (Schneider and McCann, 1970; Vijayan and McCann, 1978). Noradrenergic pathways in particular, appear to stimulate the release of luteinizing hormone (LH) (Martinovic and McCann, 1977; Hancke and Wuttke, 1979). α-Methyl-*p*-tyrosine (α-MPT) specifically inhibits tyrosine hydroxylase, the rate limiting enzyme in catecholamine biosynthesis, inhibition of that enzyme being competitive with the natural substrate tyrosine. As a result, dopamine as well as noradrenaline levels are lowered in both peripheral adrenergic tissues and in the CNS.

Abbreviations used: PE, Proestrus; E, estrus; DE, diestrus; LH, luteinizing hormone; FSH, follicle stimulating hormone; PRL, prolactin; DA, dopamine; α-MPT, α-methyl-*p*-tyrosine; L-dopa, L-dihydroxyphenylalanine; dopamine, 3, 4 dihydroxy phenylethylamine.

The activity of hypothalamic tyrosine hydroxylase (EC 1.14.16.2), the rate limiting enzyme in catecholamine biosynthesis, whose activity changes correlate well with alterations in dopamine levels as well as plasma gonadotropin and prolactin (PRL) levels, were measured during different stages of the estrous cycle and after ovariectomy. The effects of reserpine, an alkaloid which depletes brain catecholamines from storage granules, and α-MPT were evaluated by measuring plasma gonadotropin and PRL levels in order to determine if a correlation exists between the changes in hormone levels and hypothalamic tyrosine hydroxylase activity.

Materials and methods

Animals

Sexually mature, virgin, Wistar female rats weighing 180-200 g were housed under controlled conditions of light (12 h light : 12 h dark) and temperature (22±2°C). They were fed on food pellets obtained from Hindustan-Lever, Bombay, and had access to water *ad libitum*.

Vaginal smear was checked in these rats daily in the morning. Animals showing at least three consecutive 4 day cycles, were killed by decapitation at 0900, 1200 and 1700 h on the days of diestrus (DE), proestrus (PE) and estrus (E). Trunk blood was collected and plasma was separated at 4°C and stored frozen for the assay of gonadotropins and PRL. Brains were quickly removed and hypothalami were dissected out to measure tyrosine hydroxylase activity.

A group of rats were ovariectomized (OVX) under light ether anesthesia and used for experimentation 2-3 weeks later. Reserpine (Sigma Chemical Co., St. Louis, Missouri, USA. Lot 97C-0033) was dissolved in 20% ascorbic acid (10mg/0.5ml) and was administered, sc, at a dose of 10mg/kg body wt/day, for 3 days. Controls were given equal volume of vehicle. The animals were killed by decapitation, 24 h after the last injection.

α-MPT (Sigma Chemical Co., St. Louis, Missouri, USA. Lot. 58C-0371) dissolved in 0.1N HCl (200 mg/ml) (pH adjusted to 5.6-6.0 with 5N NaOH) was administered, ip, at a dose of 400 mg/kg body wt. Controls received equal volume of vehicle. Animals were decapitated 2 h after the injection. Trunk blood was collected, plasma was separated at 4°C and stored frozen for the later assay of gondatropins and PRL by radioimmunoassay (RIA).

Radioimmunoassay

Plasma LH, FSH and PRL concentrations were determined by a double antibody radioimmunoassay, using kits supplied by NIAMDD-National Institutes of Health, Bethesda, Maryland, USA, pituitary hormone distribution programme and according to the guidelines provided with the kit. The results were expressed with reference to the reference preparations, NIH-LH RP-1, FSH-RP-1, and PRL-RP-2 respectively. All samples were run in one assay, each in duplicate, to avoid interassay variation.

Hypothalamic tyrosine hydroxylase assay

Hypothalami (which include pre-optic area, medial basal hypothalamus and median eminence) were dissected out as a single block according to the procedure described earlier (Vijayan, 1974). Tyrosine hydroxylase activity was assayed by the colorimetric method of Shiman *et al.* (1971) as described earlier (Babu and Vijayan, 1981). The activity of tyrosine hydroxylase was expressed as nmol of 3,4 dihydroxyphenylalanine (dopa) formed/mg of protein/h. Protein was estimated by the method of Lowry *et al.* (1951).

Statistics: Statistical evaluation of the data was done by student's '*t*' test.

Results

Luteinizing hormone

During diestrus, the concentrations of LH were low at each time studied (0900, 1200 and 1700 h). During proestrus, however, the morning and noon values were low but by 1700 h the hormone levels were elevated significantly ($P<0.001$). There were no significant differences between the morning, noon and afternoon LH levels on estrus (figure 1).

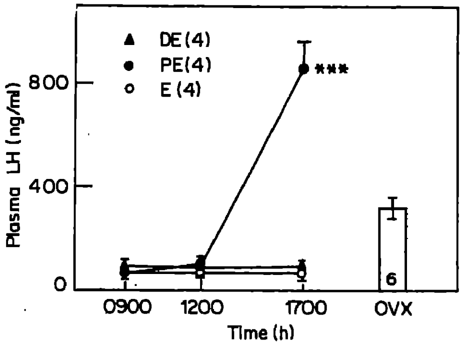

Figure 1. Plasma LH levels at different times during the estrous cycle and after ovariectomy.

In this and subsequent figures, numbers at the base of each column or in parentheses indicate the number of animals in each group and/or at each point. Vertical lines above and/or below the mean represent mean±SEM. *** $P<0.001$ *vs* 1200 PE value.

Follicle stimulating hormone

Plasma FSH levels were low at all times during diestrus. The proestrus values were low during 0900 and 1200 but at 1700 h the levels were significantly elevated ($P<0.05$) which persisted through estrus (figure 2).

Prolactin

Plasma PRL levels were low during diestrus and during the morning (0900) and noon (1200) of proestrus. There was a significant ($P<0.001$) surge of PRL during the afternoon (1700) of PE. No significant alterations in PRL levels were observed at various times (0900, 1200 and 1700 h) of estrus, though the values were slightly higher than DE and PE morning and noon concentrations (figure 3).

Tyrosine hydroxylase

Hypothalamic tyrosine hydroxylase activity was significantly higher at 1200 and 1700 h during DE, when compared to PE and E values at the same time. However,

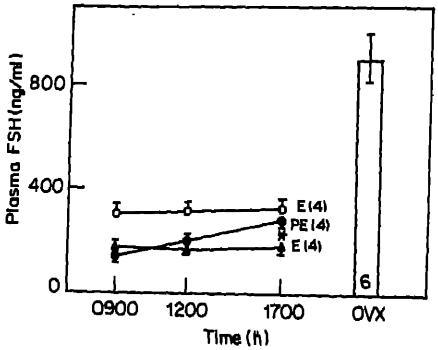

Figure 2. Plasma FSH levels at different times during the estrous cycle and after ovariectomy.

* $P<0.05$ *vs* 0900 PE levels.

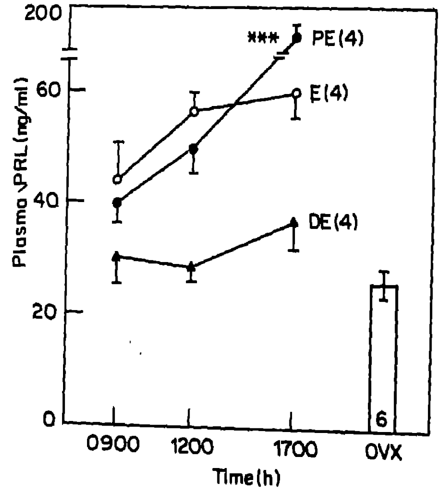

Figure 3. Plasma PRL concentrations at different times during the estrous cycle and after ovariectomy.

*** $P<0.001$ *vs* 0900 and 1200 PE levels.

there was no difference in the enzyme activity between 0900, 1200 and 1700 h during DE or E. On the contrary, the enzyme activity was significantly lowered at 1200 h during PE with respect to 0900 h or DE 1200 h value (figure 4).

Gonadotropin, PRL levels and tyrosine hydroxylase activity after ovariectomy

Plasma gonadotropin levels were significantly elevated ($P<0.001$) while PRL titers were relatively low in OVX rats (figures 1, 2 and 3). Hypothalamic enzyme activity was comparable to those of DE and E values, but significant increase in enzyme activity was evident only with respect to DE 1200 h value (figure 4).

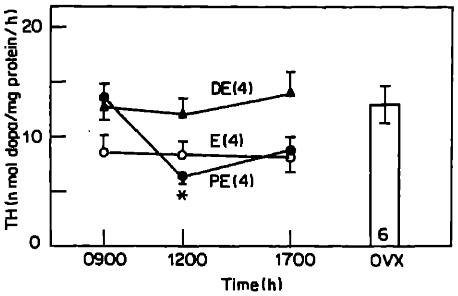

Figure 4. Hypothalamic tyrosine hydroxylase activity at different times during the estrous cycle and after ovariectomy.

* $P<0.05$ *vs* 0900 PE value and 1200 DE value.

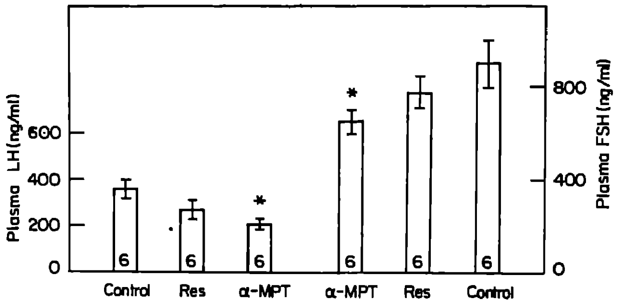

Figure 5. Plasma LH and FSH levels following the administration of reserpine or α-MPT, in OVX rats.

* $P<0.05$ *vs* control.

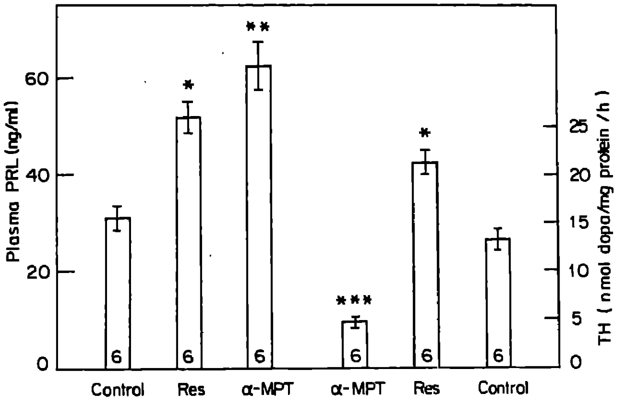

Figure 6. Plasma PRL levels and hypothalamic tyrosine hydroxylase activity following the administration of reserpine or α-MPT, in OVX rats.

* $P<0.05$ ** $P<0.01$ *vs* control *** $P<0.001$.

Gonadotropin, PRL and hypothalamic tyrosine hydroxylase activity after reserpine

Ascorbic acid diluent administration had no effect on plasma gonadotropin levels. Reserpine administration produced only a slight decrease in plasma LH and FSH levels (figure 5) while prolactin levels were significantly ($P<0.05$) elevated (figure 6).

Hypothalamic tyrosine hydroxylase activity was unaltered after the injection of the diluent. On the other hand, tyrosine hydroxylase activity was significantly elevated ($P<0.05$) following reserpine injections (figure 6).

Gonadotropin, PRL and hypothalamic tyrosine hydroxylase activity after α-MPT

Administration of α-MPT, an inhibitor of tyrosine hydroxylase, significantly suppressed ($P<0.05$) plasma LH levels at 2 h after injection in OVX animals (figure 5). Plasma FSH levels were also significantly ($P<0.05$) suppressed following α-MPT injection with respect to the control values (figure 5). α-MPT, significantly elevated ($P<0.01$) plasma prolactin levels and inhibited tyrosine hydroxylase activity (figure 6).

Discussion

Plasma LH and PRL levels during the afternoon of proestrus agree well with the earlier findings. FSH levels showed peak values during afternoon of PE, and throughout estrus. There were no significant differences in LH and PRL at different times of diestrus and estrus. Hypothalamic tyrosine hydroxylase activity showed a significant decrease at 1200 h followed by an increase of LH during the afternoon of proestrus. The decrease in tyrosine hydroxylase activity at 1200 h on PE may reflect lowered dopaminergic activity, precipitating the series of events leading to the afternoon LH surge. However, dopamine has been shown to stimulate LH release via an action of LHRH (Vijayan and McCann, 1978).

It is well accepted that DA exerts an inhibitory influence on PRL secretion. In the present study, tyrosine hydroxylase activity was significantly lowered at 1200 h on PE, which was followed by an elevation in PRL concentrations at 1700 h.

As expected, plasma LH and FSH levels were significantly elevated and PRL levels were lowered following ovariectomy. The rise in gonadotropins was obviously due to the removal of negative feedback by ovarian steroids. Since estrogens are stimulatory to prolactin release, ovariectomy resulted in decreased PRL levels.

Reserpine, a drug which blocks the uptake of catecholamines into storge vesicles, slightly suppressed plasma gonadotropin levels, but elevated PRL levels and hypothalamic tyrosine hydroxylase activity. The depletion of hypothalamic dopamine reserves by reserpine apparently elevates PRL levels. Reserpine depletes the brain of dopamine, norepinephrine and also serotonin (Shore *et al.*, 1955). The enhanced hypothalamic tyrosine hydroxylase activity is attributed to autoregulatory feedback mechanism of PRL release, by which increased levels of prolactin stimulate the synthesis of tyrosine hydroxylase. Cycloheximide, an inhibitor of protein synthesis, is reported to disrupt the PRL mediated stimulation of dopamine synthesis in these neurons (Johnston *et al.*, 1980).

When pituitary glands are incubated in the presence of reserpine, there is a significant dose-related inhibition of PRL release, suggesting that pituitary action of this alkaloid is opposite to that of CNS action, and this direct effect of reserpine to inhibit PRL release is reported to be independent on any interaction with catecholamine systems and is mediated by other, presently undefined mechanisms (Login and MacLeod, 1981).

α-MPT, a specific inhibitor of the tyrosine hydroxylase, inhibited the elevated gonadotropin levels in ovariectomized rats. Plasma prolactin levels, on the other hand, were significantly elevated. α-MPT has earlier been reported to block prevolulatory discharge of LH and progesterone induced LH release in estradiol primed rats (Kalra and McCann, 1975).

The suppression of LH and FSH release following α-MPT may be due to the inhibition of noradrenaline synthesis in different areas of brain by this compound. Noradrenaline which strongly stimulates LH release, is implicated in the physiological control of LHRH-LH release (Clifton and Sawyer, 1979). Prolactin levels were significantly elevated, apparently due to the inhibition of hypothalamic dopamine synthesis as evidenced by suppression of tyrosine hydroxylase activity. The present results provide additional evidence on the regulatory role played by the enzyme tyrosine hydroxylase and the involvement of catecholamines in pituitary gonadotropin and PRL release.

Acknowledgements

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Physico-chemical characterisation and molecular organisation of the collagen from the skin of an air-breathing fish *(Ophiocephalus striatus)**

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Abstract. Collagen has been prepared from the skin of an air-breathing Indian fish *(Ophiocephalus striatus)* by extraction with cold 0.5M acetic acid and purification by alternate precipitation with NaCl and dialysis against 0.02M Na_2HPO_4. The purified collagen was characterised with respect to physico-chemical properties, amino acid composition and chromatography of the denatured collagen. The fish collagen has a higher shrinkage temperature and denaturation temperature compared to that of the allied teleosts living in exclusively aquatic medium. These differences could possibly be reflections of the response to the rigours of the environment. As found for other vertebrate collagens, the fish collagen contains two kinds of single chains the α_1 and α_2 chains as revealed by sodium dodecyl sulphate — polyacrylamidegel electrophoresis and carboxymethylcellulose chromatography.

Keywords. Fish collagen; characterisation of collagen; chain composition.

Introduction

Previous work on the properties of the skin collagen of fishes indicates that they show variations in amino acid composition and physical properties correlated with the temperature of the environment (Takahashi and Tanaka, 1953; Gustavson, 1953, 1956). The shrinkage temperature of the skin collagen of fishes living in warmer waters was found to be higher than that of fishes living in colder waters (Gustavson, 1955). Shrinkage temperature of 63°C has been reported for the skin collagen of Australian lung fish *Neoceratodus* (Eastoe, 1957). Such a correlation between the shrinkage temperature and environmental temperature suggests that the properties of skin collagen are attributable to the mode of life. In the light of the above observations, the nature of the skin collagen of a mud-dwelling fish *Ophiocephalus* is of interest. Like *Neoceratodus* it is air-breathing and possesses accessory air-breathing organs. The nature of the biochemical adaptations of collagen in a semi-terrestrial environment is interesting. Hence a study of the physico-chemical properties and molecular organisation has been made with reference to the skin collagen of *Ophiocephalus striatus.*

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* The work reported forms part of the thesis approved by the University of Madras for the award of the Ph.D. degree to the first author.

Materials and methods

Ophiocephalus striatus used in the present investigation were collected from ponds in Madras.

Sodium dodecyl sulphate (SDS) and ammonium persulphate used were obtained from Sigma Chemical Company, St. Louis, Missouri, USA. Whatman CM-32 microgranular cellulose was obtained from Whatman Biochemicals, Kent, UK. All other chemicals were of analytical reagent grade.

Isolation and purification of collagen

The skin of *Ophiocephalus striatus* was freed of scales and adhering tissues and stirred overnight with 0.5M sodium acetate at 4°C. It was washed well with cold distilled water and extracted with 0.5M acetic acid. Purification of the extract was carried out according to the method of Piez *et al.* (1963) as outlined below. The extract was centrifuged at 18,000 rpm in a Sorvall refrigerated centrifuge for 30 min and collagen from the supernatant was precipitated by the addition of solid NaCl to a concentration of 5%. The precipitate was collected by centrifugation at 10,000 rpm and redissolved in 0.5M acetic acid. The process of precipitation with NaCl and dissolution in 0.5M acetic acid was repeated thrice and collagen in solution was dialysed against 0.02M Na_2HPO_4. The precipitate was centrifuged at 18,000 rpm for 30 min, dialysed against large volumes of 0.1M acetic acid and lyophilised.

Chemical analyses

Collagen sample was hydrolysed in 6N HCl in sealed tubes at 110°C for 24 h. Amino acid composition was determined in an amino acid analyser, Beckman Spinco model 120 C (Spackman *et al.*, 1958). Hydroxyproline content of the sample was determined by the method of Neuman and Logan (1950) and tyrosine content by the method of Ottaway (1958).

Physical properties

The shrinkage temperature (Ts) of the skin collagen was measured with a micro shrinkage meter; viscosity was measured in Ubbelhode viscometer. The denaturation temperature (Td) was obtained from the midpoint of viscosity *vs* temperature profile in the range 20 to 40°C. For determination of intrinsic viscosity, viscosity of solutions in the concentration range 0.025 to 0.15% was determined and the value was obtained by extrapolation of the specific viscosities to zero concentration.

Polyacrylamide gel electrophoresis

The procedure of Laemelli (1970) was followed using 5% (W/V) acrylamide gels. Samples were run for 4 h at 3 mA per gel and protein bands were stained with 0.1% coomassie blue in 50% TCA at 37°C for 1 h. Destaining was carried out with 7% TCA and the destained gels were scanned by using a Beckman scanning densitometer at 540 nm.

Carboxymethyl-cellulose chromatography

The method of Piez *et al.* (1963) was adopted. The collagen sample was heat denatured in 0.06M sodium acetate buffer pH 4.8 and then applied to a corboxymethyl-cellulose column (1.8 × 15 cms) maintained at 40°C which had been equilibrated with the above buffer. Elution was carried out by applying a gradient of 0-0.16M NaCl. 10 ml fractions were collected and the absorbance was measured at 230nm in a Pye Unicam model SP 1800 UV spectrophotometer.

The distribution of α and β chains in the collagen sample was determined by molecular seive chromatography using 6% agarose (Piez, 1968). A column (2.3 × 120 cm) of 6% agarose (Bio-Gel A 1.5M 200-400 mesh) was prepared and equilibrated with 0.5M Tris/1M$CaCl_2$buffer (pH 7.5). The sample (about 20 mg) was dissolved in 2-3 ml of $CaCl_2$/Tris buffer at 45°C for 5-10 min. The flow rate was maintained at 25 ml/h and 5 ml fractions were collected. The fractions were monitered at 230 nm.

Electron micrograph of the native reconstituted collagen was obtained by scanning in a Siemens Elmiskop I.

Results

About 90% of the collagen from the skin of *Ophiocephalus* could be extracted with 0.5M acetic acid which indicates a high degree of solubility. Previous work indicates that the solubility of the skin collagen of fishes varies widely. Pikkarainen (1968) observed that collagens of hag fish and ray fish could be extracted upto 80 to 90% of the total quantity. Among the bony fishes the solubility of the collagen was only 33% in pike whereas Young and Lorimer (1960) reported that about 62% of the collagen could be solubilized from full grown cod. In the case of *Ophiocephalus* the solubility of the skin collagen remains unchanged at all growth stages which is in contrast to the mammalian collagens in which the solubility decreases with age (Bailey, 1967).

Table 1. Aminoacid composition of skin collagen of *Ophiocephalus*.

Amino acid	*Ophiocephalus* skin collagen	Calf skin collagen
	Residues/1000	
3-Hydroxyproline	—	—
4-Hydroxyproline	85.0	99.1
Aspartic acid	42.0	46.1
Threonine	24.8	19.4
Serine	38.0	36.7
Glutamic acid	67.0	67.9
Proline	125.0	128.2
Glycine	323.0	322.3
Alanine	116.3	109.2
Valine	22.8	24.1
Methionine	15.1	6.7
Isoleucine	9.7	11.5
Leucine	22.5	24.5
Tyrosine	2.8	3.1
Phenylalanine	11.1	10.2
Hydroxylysine	7.1	7.5
Histidine	6.1	5.4
Lysine	27.8	28.7
Arginine	53.8	49.4

The results of the amino acid analysis are given in table 1. The amino acid content of 210 residues/1000 resembles that reported for the skin collagen of *Neoceratodus* (Eastoe, 1957). The value is on the higher side when compared to those of teleosts such as carp and pike and hake (Eastoe, 1967; Yamaguchi *et al.*, 1976). A similar accord with skin collagen of *Neoceratodus* in the values for serine and threonine was noted. The significance of the higher values for these residues is not clear although it has been suggested by Pikkarainen (1968) that the higher content of serine and threonine may provide more hydroxyl groups to make up for the deficiency in the content of hydroxyproline.

The physical properties of skin collagen of *Ophiocephalus* are given in table 2. A shrinkage temperature of 60°C was observed which is high when compared to the other aquatic teleosts but resemble *Neoceratodus*. In the case of pike classified as a warm water fish, the shrinkage temperature has been reported to be 55°C while that of the allied type cod living in cold waters at a temperature of 15°C a lower shrinkage temperature such as 40°C has been observed (Gustavson, 1955). The denaturation temperature of 32.9°C (figure 1) is also higher than the values for aquatic teleosts. Rigby and Prosser (1975) observed that the melting temperature

Table 2. Physical properties of skin collagen of *Ophiocephalus*.

Properties	*Ophiocephalus* skin collagen
Shrinkage temperature °C	60.0
Denaturation temperature °C	32.9
Reduced viscosity dl/g	22.0
Intrinsic viscosity	15.2

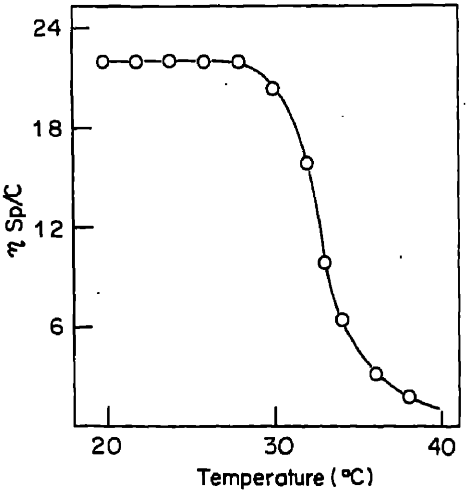

Figure 1. Denaturation curve of acid soluble collagen of skin of *Ophiocephalus* based on viscosity measurements at different temperatures.

of collagen in the skin of fishes from different depths have a correlation with the temperature of external medium of fish. The values for both reduced viscosity and intrinsic viscosity (figure 2) of the collagen resemble those of other fish collagens.

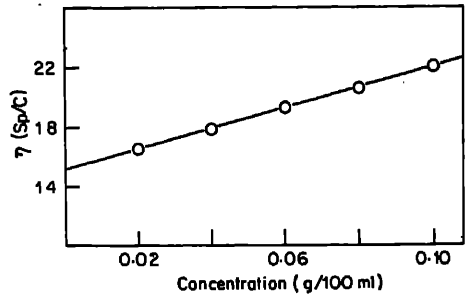

Figure 2. Reduced viscosity *vs* concentration plot for skin collagen of *Ophiocephalus* (in 0.1M acetic acid at pH 3.6).

The subunit composition was determined after denaturation of the samples and by subjecting it to polyacrylamide gel electrophoresis (figure 3). The pattern comprises of 2α chains corresponding to α_1 and α_2 chains of calf skin collagen

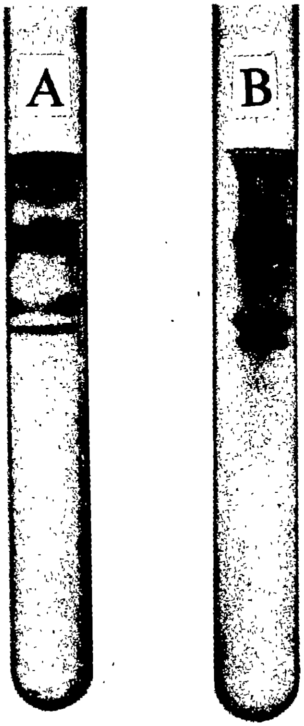

Figure 3. 5% Sodium dodecyl sulphate—polyacrylamide gel electrophoretic pattern. **A.** *Ophiocephalus* skin collagen. **B.** Calf skin collagen.

together with β and γ components. The results were confirmed by carboxy methyl cellulose chromatography (figure 4). The results obtained show the presence of both α_1 and α_2 chains. The trailing edge of α_1 peak was found to contain β_{11} and the peak preceding the α_2 peak was found to be a mixture of β_{12} and α_2. It is seen that the chain composition of skin collagen of *Ophiocephalus* conforms to the pattern of Type I collagen of vertebrates.

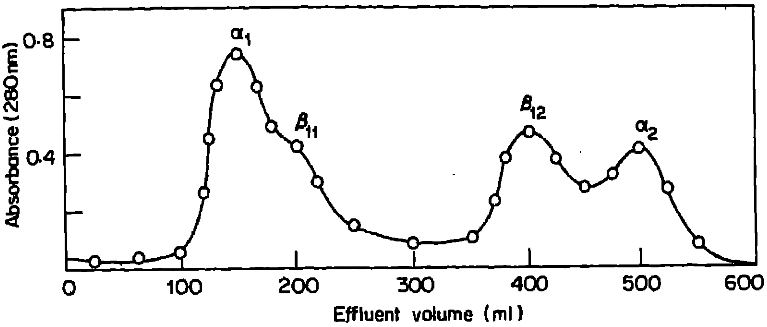

Figure 4. Carboxy methyl—cellulose elution pattern of denatured acid soluble collagen of skin of *Ophiocephalus*.

The elution profile of the denature skin collagen of *Ophiocephalus* from 6% agarose is shown in figure 5A. The α-components as well as the dimeric and trimeric

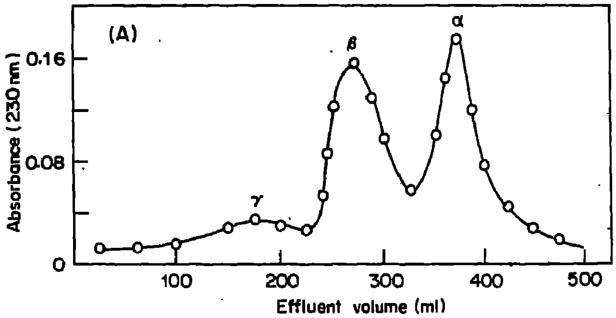

Figure 5A. Elution profile of denatured acid soluble collagen of skin of *Ophiocephalus* on 6% agarose.

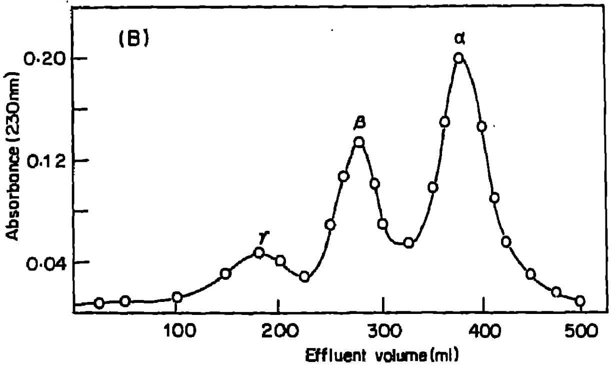

Figure 5B. Elution profile of denatured acid soluble calf skin collagen on 6% agarose.

components eluted in the region corresponding to those of calf skin collagen (figure 5B). The α-chains of skin collagen of *Ophiocephalus* thus have a molecular weight of 95,000-100,000 similar to those reported for other fish collagens.

The electron micrograph of the skin collagen of *Ophiocephalus* shows a banding pattern of 640 Å (not shown) which accords with the pattern reported for vertebrate collagens.

Discussion

The results reported above indicate that the skin collagen of *Ophiocephalus* is peculiar in a number of respects particularly in regard to the amino acid composition and some of its physical properties such as shrinkage temperature (Ts) and denaturation temperature (Td). A higher amino acid content (proline+hydroxyproline) is noted on comparison with skin collagens of teleosts living exclusively in aquatic medium. Correlated with this feature of amino acid composition are the physical properties of skin collagen with Ts and Td of 60°C and 32.9°C respectively. These values are also higher than those reported for skin collagen of allied teleosts such as pike, carp and cod. A higher Ts value has been related to environmental temperatures of fishes in which they live. Although a correlation between Ts values and temperature of the environment has been supported by the results of various studies carried out on skin collagen of fishes, it is seen that the Ts values extend over a range of 15-30°C above the maximal environmental temperature. It is therefore suggestive that it is not merely a question of approximating to the temperature of the environment but has probably a deeper biological significance associated with metabolic factors involving collagen synthesis and function.

In this context it is relevant to recall the data on the physical properties of *Neoceratodus* which is typically air-breathing (Eastoe, 1957). The Ts value approximates to that reported in the present study on the skin collagen of *Ophiocephalus*. The air-breathing habit is common to both fishes and may suggest that this may be one of the factors contributing to the high values for Ts noted in them. It may be suggested that the high content of iminoacids and the correlated high values for Ts and Td are of the nature of responses to the rigours of the environment and hence of adaptive value. The mode of life of this fish is unusual and lives burried under mud for prolonged periods. Necessarily such a mode of life for a fish would demand adjustments and modifications in the metabolic pattern which may be reflected in the biochemistry of skin collagen.

Notwithstanding the deviations in amino acid compositon and physical properties of skin collagen of *Ophiocephalus* reported above, there is seen a close similarity in its chain composition to that of other teleosts such as carp, pike and hake in conforming to Type I collagen of higher vertebrates. (Piez *et al.*, 1963; Paz *et al.*, 1967; Pikkarainen, 1968). In the case of cod fish, Piez (1965) reported the presence of three non-identical α chains. It is seen that the molecular organisation which is determined by genetic factors remains unaltered to a large extent and is not influenced by factors external to the organism but changes in amino acid composition and physical properties of collagens have been reported to occur in response to environmental demands.

Acknowledgement

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Fluorescence studies on the interaction of some ligands with carcinoscorpin, the sialic acid specific lectin, from the horseshoe crab, *Carcinoscorpius rotundacauda*

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Abstract. The binding affinities of some ligands towards the sialic acid-specific lectin carcinoscorpin, from hemolymph of the horseshoe crab *Carcinoscorpius rotundacauda* have been determined by protein fluorescence quenching in presence of ligands. Among the ligands studied, the disaccharide O-(N-acetylneuraminyl)-(2 → 6)-2-acetamido-2-deoxy-D-galactitol has the highest K_a (1.15×10^6 M^{-1}) for carcinoscorpin. Studies on the effect of pH on K_a values of disaccharide suggests the possible involvement of amino acid residues having pK_a values around 6.0 and 9.0 in the binding activity of carcinoscorpin. There were distinct changes in the accessibility of the fluorescent tryptophan residues of carcinoscorpin by ligand-binding as checked through potassium iodide quenching.

Keywords. Horseshoe crab lectin; sialic acid; fluorescence; ligand-binding.

Introduction

Lectins are good analytical tools to undertake diverse studies on cell-membranes and to isolate and characterize specific cell-surface glycoconjugates (Lis and Sharon, 1977). They have served as good models to study protein-carbohydrate interactions (Goldstein and Hayes, 1978). Many of the physical and biological functions of oligo-, polysaccharides, glycoproteins and glycolipids occurring in the body fluids and in the structural elements of cells have been attributed to their sialic acid components (Lloyd, 1975). Although lectins with specificity towards sialic acid have not been as well characterized as those with specificity for other sugar residues such as D-galactose, D-mannose, D-glucose, N-acetylglucosamine, etc. (Goldstein and Hayes, 1978), a few sialic acid specific lectins have been reported — limulin from the horseshoe crab *Limulus polyphemus* (Marchalonis and Edelman, 1968); a lectin isolated from the serum of the lobster, *Homarus americanus* (Hall and Rowlands, 1974); an agglutinin from the albumen gland of certain snails (Voigtmann *et al.*, 1971); a protein extracted from the prickly lettuce plant, *Lectura scariola* (Yen *et al.*, 1980); and our laboratory has also isolated a sialic acid-specific lectin, carinoscorpin, from the hemolymph of the marine horseshoe crab,

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Abbreviations used: Ca^{2+}-free carcinoscorpin, Carcinoscorpin dialysed exhaustively against 0.05 M Tris-HCl, pH 8.0 containing 0.1 M NaCl; EDTA-carcinoscorpin, carcinoscorpin dialysed exhaustively against 0.05 M Tris-HCl, pH 8.0 containing 0.1 M NaCl and 0.01 M Na_2-EDTA; NeuNAC, N-acetylneuraminic acid.

Carcinoscorpius rotundacauda (Bishayee and Dorai, 1980). Information on the interaction of this lectin with ligands and the specificity towards 2-oxo-3-deoxyoctonate and some physical aspects of its binding with sialoglycoproteins have been reported recently (Dorai *et al.*, 1982; Mohan *et al.*, 1982).

In view of the possible use of carcinoscorpin in the study of cellular sialoglycoconjugates, it was essential to characterize the mechanism of binding of this lectin with its ligands. Physicochemical methods provide a better resolution of binding specificities than inhibition of hemagglutination which is semi-quantitative. We report in this paper the fluorescence studies of carcinoscorpin binding with various ligands.

Materials and methods

N-Acetylneuraminic acid (NeuNAc), N-glycoloylneuraminic acid, 2-oxo-3-deoxyoctonate, D-glucuronic acid, neuraminyl lactose and phosphorylcholine (Ca^{2+}-salt) were purchased from the Sigma Chemical Company, St. Louis, Missouri, USA. All other chemicals used were of the analytical grade.

Purification of carcinoscorpin

The sialic acid binding lectin, carcinoscorpin of *Carcinoscorpius rotundacauda* hemolymph was purified to homogeneity by the procedure of Dorai *et al.* (1981). Purified carcinoscorpin was kept in small aliquots at a protein concentration less than 2 mg/ml and stored at −20°C until further use.

Preparation of carcinoscorpin-specific disaccharide

The disaccharide, O-(N-acetylneuraminyl)-(2 → 6)-2-acetamido-2-deoxy-D-galactitol, was prepared from sheep submaxillary mucin as described earlier (Mohan *et al.*, 1982).

Fluorescence measurements (ligand titration)

The ligand-induced quenching of protein fluorescence was studied in an Aminco-Bowman spectrofluorometer at 25°C. The association constants (K_a) for various ligands were determined according to Jolley and Glaudemans (1975). Carcinoscorpin solution of fixed protein concentration (0.1×10^{-6} M; A_{280} 0.05) in 0.05 M Tris-HCl, pH 8.0 containing 0.1 M NaCl and 0.01 M $CaCl_2$ was titrated with the ligands of appropriate concentration. The excitation wavelength was 280 nm and the quenching of fluorescence intensity was measured at 332 nm at which it was maximal. The buffer solution and the ligand solutions did not have any fluorescence between 290 nm and 400 nm. The spectra of ligand-carcinoscorpin solution after completion of titration was recorded for each ligand.

The free energy change of binding for each ligand was calculated by using the question, $\Delta G^\circ = -RT\ln K_a$.

Effect of pH on K_a

Titration of native carcinoscorpin with disaccharide was done as described above at different pHs (4.5-9). The log values of K_a were plotted against pH.

Accessibility of tryptophan residues

The accessibility of tryptophan residues in the presence and absence of ligands (disaccharide and phosphorylcholine) was studied by KI quenching. Native lectin (0.93×10^{-7}M) and lectin in presence of the ligands, disaccharide (8×10^{-6}M) and phosphorylcholine (0.01 M), were titrated with 5 M KI, the addition being done in small aliquots. KI solution contained 200 mM sodium thiosulphate to suppress tri-iodide formation. A control titration with 5 M NaCl was performed simultaneously for non-specific changes in relative fluorescence intensity due to ionic-strength variation. After correcting for dilution effect, the fluorescence intensity data was plotted as F_0/F against [KI] where F_0 and F are the fluorescence intensities of native and ligand-bound carcinoscorpin, respectively, of same protein concentration.

Determination of protein

The concentration of lectin solution was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard.

Results and discussion

Emission spectra of carcinoscorpin, Ca^{2+}-free carcinoscorpin and EDTA carcinoscorpin

Since Ca^{2+} is a prerequisite for the biological activity of carcinoscorpin (Mohan *et al.*, 1982) the effect of Ca^{2+} on the structure of carcinoscorpin was checked by fluorometry. The emission spectra of carcinoscorpin and its Ca^{2+}-free derivatives were recorded (figure 1). The fluorescence intensity at the emission maximum (332 nm) is lower in case of Ca^{2+}-free carcinoscorpin and EDTA-carcinoscorpin compared to the native protein indicating a change in the micro-environment of fluorescent aromatic amino acid residues upon removal of Ca^{2+} from the lectin.

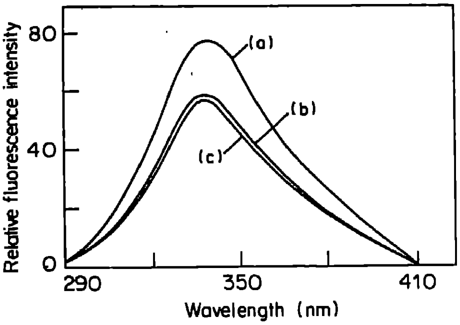

Figure 1. *Fluorescence spectra of native carcinoscorpin, Ca^{2+}-free-carcinoscorpin and EDTA-carcinoscorpin.*

The emission spectra of native carcinoscorpin, Ca^{2+}-free-carcinoscorpin and EDTA-carcinoscorpin (see 'Abbreviations') was measured at 25°C in 0.05 M Tris-HCl pH 8.0 containing 0.1 M NaCl. Protein concentration was 0.1×10^{-6} M in each case. Excitation wavelength was 280 nm (a) Native carcinoscorpin (b) Ca^{2+}-free-carcinoscorpin (c) EDTA-carcinoscorpin.

From circular dichroism measurements, Roche *et al.* (1978) have reported changes in the structure of limulin in absence of Ca^{2+}. With the addition of 0.01 M Ca^{2+} the fluorescence intensity of Ca^{2+}-free carcinoscorpin however did not increase. The decrease in fluorescence intensity upon removing Ca^{2+}, therefore, does not seem to be reversible. But full biological activity (as checked by binding to fetuin) could be restored in Ca^{2+}-free-carcinoscorpin or EDTA-carcinoscorpin with the addition of 0.01 M Ca^{2+} (Mohan *et al.*, 1982). This is corroborated by the findings that disaccharide could quench, in presence of 0.01 M Ca^{2+}, the fluorescence intensity of Ca^{2+}-free and EDTA-carcinoscorpin to the same extent as that of native carcinoscorpin (see "binding parameters for ligands"). The observation suggests a structure for carcinoscorpin that could tolerate minor perturbations without losing the ability to regain full biological activity.

Binding parameters for ligands

In the presence of some ligands, the fluorescence intensity of carcinoscorpin decreased by 8-12% and the emission maximum (332 nm) underwent red shift of 5 nm. Taking advantage of this quenching the K_a values of various ligands for carcinoscorpin were calculated (table 1). A representative Scatchard plot for the binding of disaccharide and NeuNAc is given in figure 2. The higher K_a value of disaccharide (approximately 100 times than that of NeuNAc) reflects the high specificity of carcinoscorpin for the disaccharide which is complemented by our earlier studies on hemagglutination (Mohan *et al.*, 1982). While screening various ligands to inhibit carcinoscorpin-fetuin interaction and also hemagglutination by carcinoscorpin, it was noted that apart from NeuNAc and the disaccharide, D-glucuronic acid and 2-oxo-3-deoxyoctonate were also good inhibitors (Mohan *et al.*, 1982) which promoted us to determine the K_a values for these ligands. The association constant for D-glucuronic acid is found to be 3.64×10^3 M^{-1}. But

Table 1. Binding constants (K_a), maximal fluorescence change (ΔF_{max}) and free energies of binding ($-\Delta G^o$) for various ligands with carcinoscorpin.

Ligands	K_a (M^{-1})	$-\Delta G^o$ (KJ/mol)	ΔF_{max} (%)	Minimum concn. needed for inhibition of one hemagglutination dose (mM)[a]
N-Acetylneuraminic acid	8.56×10^3	2.53	10	1.0
N-Glycoloylneuraminic acid	3.6×10^3	20.37	13	0.5
2-Oxo-3-deoxyoctonate	ND[b]	—	<2	0.84
D-Glucuronic acid	3.64×10^3	20.40	7	0.8
N-Acetylneuraminyl galactitol	1.15×10^6	34.72	12	0.002
N-Acetylneuraminyl lactose	6.2×10^3	21.73	8.5	0.12
Phosphorylcholine	ND[b]	—	<3	10.0

[a] Values taken from Mohan *et al.* (1982).

[b] Not determined.

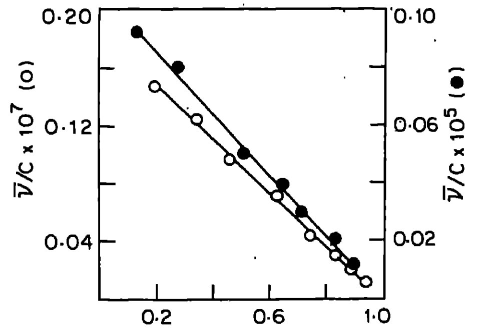

Figure 2. *Scatchard plots of the binding data of carcinoscorpin titrated with ligands.*

Carcinoscorpin solution (0.1×10^{-6} M, A_{280} 0.05) in 0.05 M Tris-HCl pH 8.0 containing 0.1 M NaCl and 0.01 M $CaCl_2$ was titrated at 25°C with disaccharide (○) and NeuNAc (●). The left side ordinate is for the disaccharide and right side ordinate is for NeuNAc.

unfortunately too low a quenching (<2.0%) of carcinoscorpin fluorescence was observed in the presence of 2-oxo-3-deoxyoctonate and hence its K_a could not be determined. However, in presence of 0.01 M 2-oxo-3-deoxyoctonate, the K_a of the disaccharide became 0.9×10^5 M^{-1}, a decrease of 10 fold of the value found in absence of 2-oxo-3-deoxyoctonate. At this stage, it is only possible to suggest that 2-oxo-3-deoxyoctonate binds at a site contiguous to (overlapping site) or the same site as that of the disaccharide but with a different orientation.

Recently Robey and Liu (1981) have shown that limulin, a sialic acid-specific lectin, could bind to phosphorylcholine which is at a site different from that of sialic acid binding site. Our earlier results showed that phosphorylcholine does not inhibit the binding of carcinoscorpin to sialoproteins or its hemagglutinating activity (Mohan *et al.*, 1982) which does not, however, rule out its binding to carcinoscorpin. When we measured the fluorescence of carcinoscorpin in presence of phosphorylcholine, a very small decrease in fluorescence intensity was observed (table 1) similar to that seen with 2-oxo-3-deoxyoctonate. However, unlike 2-oxo-3-deoxyoctonate, phosphorylcholine, even at concentration as high as 0.1 M does not alter the K_a of carcinoscorpin for disaccharide. The result does not establish whether phosphorylcholine is capable of binding to carcinoscorpin. But it suggests that, similar to limulin, the binding site for phosphorylcholine, if there is any in carcinoscorpin, is different from that for the sialic acid derivatives.

pH dependence of binding

The variation in the K_a values with pH is shown in figure 3. The optimum pH for binding was found to be around pH 8.0. We could not observe any intrinsic fluorescence quenching by the disaccharide above and below the pHs 9.0 and 5.0, respectively, suggesting the lack of interaction of lectin with the ligand around these extremes of pHs. The lectin, however, could regain its binding activity, as observed through fluorescence quenching, when brought back to pH 8.0 from pH 9.0 and pH 5.0. The ionizing groups at pK_a around 6.0 and 9.0 are possibly involved in the binding process. It is relevant to note that his residues (pK_a 6.0) have been proposed to be involved in the Ca^{2+} binding site of Concanavalin A (Hardman and

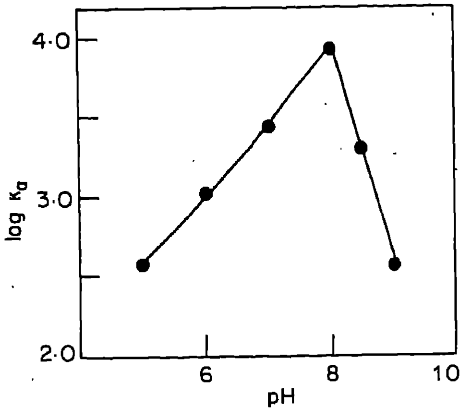

Figure 3. *Effect of pH on the binding of disaccharide to carcinoscorpin*

Titration of native carcinoscorpin (0.93×10^{-7} M) in 0.05 M Tris-HCl, 8.0 containing 0.1 M NaCl and 0.01 M $CaCl_2$ was done at different pHs with disaccharide. The K_a values calculated in each case were plotted as their log values against pH.

Goldstein, 1977). Chemical modification studies of carcinoscorpin employing group-specific reagents should help in identifying the amino acid residues required for binding Ca^{2+} and/or sialic acid.

KI quenching

The accessibility of tryptophan residues in proteins and the effect of ligand on the accessibility of tryptophan could be studied through iodide quenching of protein

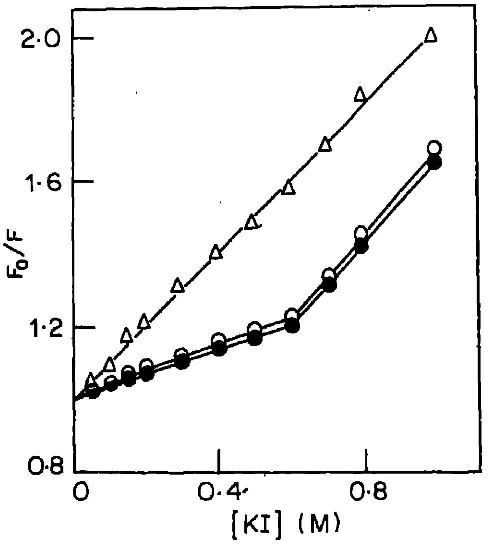

Figure 4. *KI quenching of carcinoscorpin in the presence and absence of disaccharide.*

Stern-Volmer plot of the quenching of carcinoscorpin (0.93×10^{-7} M) in 0.05 M Tris-HC , pH 8.0 containing 0.1 M NaCl and 0.01 M $CaCl_2$ with 5 M KI. Excitation and emission wavelengths were 280 and 332 nm, respectively. (●) native carcinoscorpin alone, (○) native carcinoscorpin in presence of 0.01 M phosphorylcholine, (Δ) native carcinoscorpin in presence of disaccharide (8×10^{-6} M).

fluorescence (Lehrer, 1967). This method has been utilized to probe the effect of the disaccharide binding on carcinoscorpin (figure 4). The Stern-Volmer plot of KI quenching data revealed difference in accessibility among the fluorescent tryptophan residues of carcinoscorpin in the absence of ligand (figure 4, ●). In presence of the disaccharide, interestingly, all the tryptophans behaved as a single homogeneous class, and a substantial increase in the accessibility compared to the unliganded carcinoscorpin was noticed (figure 4, Δ). The tryptophan residues of low accessibility in unliganded lectin seems to be perturbed by disaccharide with the effect that these residues acquired higher accessibility. The disaccharide could do so, either by directly binding in the vicinity of tryptophan residues and/or, through a change in the conformation of carcinoscorpin structure subsequent to ligand binding. However, in the presence of phosphorylcholine, no change in the accessibility of tryptophan residues was observed, and the plot was coincident with that of the lectin not combined with the ligand (figure 4, ○). The quenching data of other lectins like wheat germ agglutinin and *Abrus precatorius* seed agglutinin showed that ligand decreased the accessibility of fluorescent residues in them (Privat and Monsigny, 1975; Herman and Behnke, 1980). But binding of lactose to *Momordica charantia* lectin caused a slight increase in the accessibility of tryptophan residues but without affecting the pattern of heterogeneity (Das *et al.*, 1981).

To summarize, we have characterized the binding of disaccharide with carcinoscorpin through fluorescence measurements. Due to low changes in fluorescence, K_a values for 2-oxo-3-deoxyoctonate and phosphorylcholine could not be determined. However, it is inferred that 2-oxo-3-deoxyoctonate could bind at the same site as that of disaccharide or to an overlapping site with a possible difference in its orientation. In contrast, phosphorylcholine, if at all it binds to carcinoscorpin, would bind at a site different from that of sialic acid derivatives.

Acknowledgements

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Effect of sealing on the incorporation of pyrene in goat erythrocyte ghosts – A fluorescence study

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Abstract. The incorporation of pyrene within the membrane interior of goat erythrocyte ghost has been estimated from its fluorescence spectrum. The excimer to monomer fluorescence intensity ratio of embedded pyrene is a function of the fluidity of its environment and the magnitude of its incorporation. Our study shows that this ratio is considerably less (30%) in a pre-sealed ghost than in the non-sealed ghost revealing that the site of incorporation of the probe is indeed the hydrophobic interior of the membrane; as in the later case, the probe has access to the membrane interior from both sides of the membrane. Our study on kinetics of molecular exchange indicates a very fast (of the order of seconds) transfer rate of pyrene from probed to unprobed erythrocyte ghosts through the aqueous phase rather than actual fusion of the membranes.

Keywords. Erythrocyte ghosts; probe incorporation; excimer fluorescence.

Introduction

Spectroscopic probes have long been used in the development of our knowledge of cells and membranes (Vanderkooi and Callis, 1974; Galla and Sackman, 1974; Morrisett *et al.*, 1975; Dembo *et al.*, 1979; Georgescauld *et al.*, 1979; Flamm *et al.*, 1982). Probes of different polarity can be incorporated in different parts of the membrane. We have used pyrene, an excimer forming fluorescent probe, to study the fluidity picture within the goat erythrocyte ghost membrane. The probe pyrene has already been used by several workers both in model membranes as well as in biological membranes (Galla and Sackman, 1974; Morrisett *et al.*, 1975; Dembo *et al.*, 1979; Georgescauld *et al.*, 1979; Flamm *et al.*, 1982; Sengupta *et al.*, 1976, 1982). The single most important characteristic feature of this lipophilic, non-ionic probe pyrene is its ability to form short-lived dimers in the excited state. The ratio of the monomer (I at $\lambda = 390$ nm) to excimer intensity (I' at $\lambda' = 485$ nm) of the embedded pyrene when illuminated at 320 nm, is an index of fluidity parameter of its environment and also of its magnitude of incorporation (Galla and Sackman, 1974).

Pyrene being apolar in nature, its natural location is in the hydrophobic lipid region of the membrane. In our study with the goat erythrocyte ghosts, it is observed that the probe incorporation is more in the non-sealed ghost compared to that in the pre-sealed ghost. This implies that the probe has access both to the outer and inner leaflets of the erythrocyte membrane in case of the non-sealed ghost whereas for the pre-sealed ghost only the outer leaflet is accessible. The kinetics of mixing between probed and unprobed ghosts reveal a fast transfer of probe rather than actual fusion of the membranes, which is supposed to be a slow process (Sengupta *et al.*, 1976).

Materials and methods

Glass distilled water, oxygen-free buffer, zone-refined pyrene, analytical-grade chemicals and spectral grade solvent had been used. Fluorescence measurement was made with Carl-Zeiss spectrofluorometer (model No. ZFM/4C). Sample temperature was kept constant (±0.5°C) by circulating water at a particular temperature through the sample holder chamber of the spectrofluorometer.

Fresh blood from goat was collected from the local market in 168 mM NaCl solution (normal saline) with 0.35% sodium citrate as anticoagulant.

Isolation of erythrocyte ghost

Goat blood sample was centrifuged at 1000 g for 20 min. Buffy coat was removed by aspiration and by resuspending the pellet three times in normal saline and centrifuging at 1000 g for 20 min. The pellet which contained only red blood cells was then haemolysed by adding 50-60 times volume of 10 mM NaCl solution at 0°C. Good frothing indicated completion of haemolysis. Cell debris was removed by centrifuging at 1000 g for 20 min and the pellet was discarded. The supernatant was centrifuged at 20,000 g for 40 min. The pellet was washed two more times in 10 mM NaCl solution. The pink jelly-like pellet contained non-sealed ghost (Marchesi, 1974). All the operations were carried out at 4°C. Protein content was estimated by the method of Lowry *et al.* (1951).

For sealing, to 1 ml of non-sealed ghost suspension in 10 M NaCl solution, 0.78 ml of 1 mM NaCl solution was added. The solution was then stirred for 15 min at 0°C, to give sealed ghost in 168 mM NaCl solution (Zwaal *et al.*, 1975).

Incorporation of pyrene

Using various concentrations of pyrene and different times and temperature of incubation, it was found that the optimum condition for pyrene incorporation involved incubation with 0.3 mg pyrene/5 mg of protein at 37°C for 2 h (data not shown). A thin film of pyrene (0.3 mg) was made at the bottom of a round bottom flask by spreading its chloroformic solution and then evaporating the solvent under nitrogen atmosphere (Morrisett *et al.*, 1975). The probe was incorporated as follows:

(a) *In non-sealed ghost:* 5 ml of non-sealed ghost suspension containing 5 mg of protein in 10 mM NaCl solution was added to the pyrene film. The mixture was then incubated by stirring constantly at 37°C for 2 h. Unbound pyrene was removed by centrifugation at 1000 – 2000 g, followed by dialysis. Supernatant was used for fluorescence measurement.

(b) *In pre-sealed ghost:* 5 ml of the pre-sealed ghost suspension containing 5 mg of protein in normal saline was added to the pyrene film. The rest of the procedure was as above.

Pyrene exchange study

Equal volumes of pyrene-probed and unprobed sealed ghost suspension were mixed and the fluorescence spectrum was taken immediately after mixing. The peak intensities were monitored at intervals of 1, 2, 5 and 10 min. The whole spectrum was taken once again after a steady state had been reached. The experiment was carried out at 37°C.

Results

Pyrene-incorporated membrane preparation, when illuminated at 320 nm, shows a highly resolved monomer of intensity I′ at 390 nm and a broad excimer emmission band of intensity I′ at 485 nm (figure 1a). The excimer formation being a diffusion-controlled process, this ratio I′/I is an indicator of the fluidity picture of its environment. At a constant temperature, however, the ratio is directly proportional to the effective concentration of the probe incorporated within the membrane. Comparison of I′/I for pre-sealed ghost and non-sealed ghost shows that pyrene incorporation in the previous case is about 30% less than that in the later (I′/I — 1.8 for pre-sealed ghost and 2.33 for non-sealed ghost).

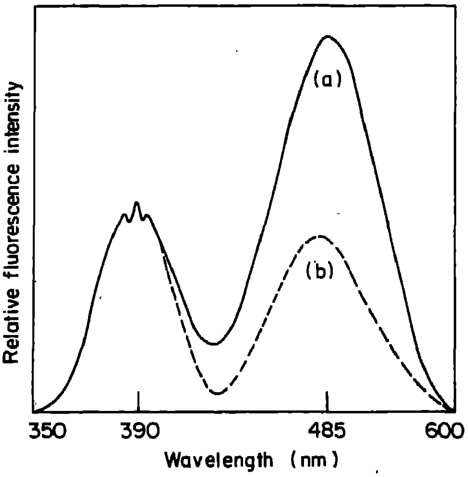

Figure 1. Kinetics of probe transfer from probed to unprobed sealed erythrocyte ghosts. Sample temperature 37°C.

(a) Spectrum of pyrene-probed erythrocyte sealed ghost before mixing.
I′/I — 1.8.
(b) Spectrum of the mixture of equal volumes of probed and unprobed sealed erythrocyte ghost immediately after mixing.
I′/I — 0.89, value remains unchanged for successive readings at 1, 2, 5 and 10 min intervals (only the peak intensities were monitored).

The kinetics of molecular exchange between ghost membranes have been studied by rapid mixing of equal volumes of pyrene-probed and unprobed sealed ghost membrane. I′/I reduces to half of its original value immediately after mixing (figure 1b) and remains unchanged upto intervals of 1, 2, 5 and 10 min.

Discussion

Pyrene being non polar, its natural site of incorporation will be the lipid mileau of the membrane. The comparison of its incorporation in sealed and non-sealed ghosts shows that this indeed is the situation. Pyrene approaches the hydrophobic interior both from inside and outside the membrane surface in case of non-sealed ghost, whereas it has access only from outside in case of pre-sealed ghost. Result shows that incorporation in non-sealed ghost is indeed 30% more than that in the sealed ghost.

It has been shown by several workers (Sengupta *et al.*, 1976; Thilo, 1977) that label exchange between phospholipid bilayers is a fast process and that actual fusion, which is a very slow process takes place only under special condition. In our kinetics study, we verify that rapid transfer of probe takes place between probed and unprobed sealed erythrocyte ghost membrane. The ratio I′/I of the mixture of equal volumes of probed and unprobed membrane reduces to half of its original value immediately after mixing remains unchanged. This indicates a very rapid transfer of the membrane bound pyrene.

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Comparative study of major lipid constituents in human and monkey aorta

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Abstract. The contents of total lipids, phospholipids, cholesterol, triglyceride and free fatty acid in intima and media of thoracic and abdominal segments of aorta from normal human subjects and rhesus monkeys were determined. An increase in total lipids in intima as compared to that of media was noted in both species. A comparison of lipid contents of thoracic and abdominal segments from both species revealed that abdominal segment contained significantly greater lipid. Further, human thoracic and abdominal aortic intima and media had higher content of cholesterol as compared to that of monkey aorta. The differences in lipid profile in aorta of these two species have been highlighted to provide a clue with respect to the differences in the prevalence and morphology of atherosclerosis as seen in monkey and man.

Keywords. Neutral and phospholipids; human and monkey aorta.

Introduction

Man and subhuman primates are phylogenetically related and present similarity in the distribution and morphological features of naturally occurring atherosclerosis (Strong and Tappen, 1965; Lindsay and Chaikoff, 1966; Chawla *et al.*, 1967; Chakravarti *et al.*, 1976; Chakravarti and Kukreja, 1981). However, frequency incidence and the prevalence of this disease in wild macaques and baboons is much lower than seen in human beings (Middleton *et al.*, 1967; Strong *et al.*, 1976; Chakravarti and Kukreja, 1981). The causes of this difference in the frequency incidence could be many, but among them the lower level of serum cholesterol in macaques as compared to that of man may play an important role (Portman and Alexander, 1966; Chawla *et al.*, 1967). Other factors like differences in arterial lipids and structural proteins between the two species may be contributing significantly to this difference. In view of the above, it was thought worthwhile to make a comparative study of normal profile of the aorta of man and rhesus monkey which may give a clue to the differences observed in the frequency incidence and biochemical characteristics of atherosclerosis in these two species.

Materials and methods

Collection of aorta samples and separation of intima and media

Aorta samples were collected from apparently normal healthy male. rhesus monkeys and from 11 young male human subjects who died of street accidents. The body weight of rheusus monkeys ranged between 4-5 kg which gave an approximate estimate of age as 4 years and for human beings the age range was between 25-35 years. In monkeys, the clinical parameters including blood pressure, electrocardiogram, ocular fundus and serum lipids and lipoproteins were within normal limits. Tuberculin test was done to exclude tuberculosis. Parasitic infection was excluded by routine stool examination. The whole aorta was dissected out, opened longitudinally and examined with a hand lens. Those samples which did not reveal macroscopic and microscopic lesions were taken for this study. The upper 2/3rd of the thoracic aorta starting from first intercostal artery opening was removed to study the lipid profile. Similarly, the upper 2/3rd of the abdominal aorta starting from coeliac artery was taken for lipid study. The aorta samples were fixed on a waxed tray with endothelial surface facing upwards, and the intima was scrapped off with the back of a scalpel handle. In the case of monkeys, separation of intima from media was difficult since the intima was very thin, but in the case of humans the intima could be easily scrapped off from media (Smith *et al.*, 1967).

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Extraction, separation and estimation of lipids

Lipid extraction from aorta samples was done by the method of Folch *et al.* (1957) using chloroform-methanol (2:1, v/v) mixture; the solvents were redistilled before use. Triglycerides, free fatty acids, free and esterified cholesterol were separated by using single dimension thin layer chromatography. Phospholipid fractions were separated by double dimension thin layer chromatography. Free and esterified cholesterol were estimated by the method of Zlatkis *et al.* (1953), triglycerides by Van Handel and Zilversmit (1957), phospholipid phosphorus by Bartlett (1959) and free fatty acids by the technique of Dole (1956).

Results

Table 1 shows the values of different components of neutral lipid i.e. total cholesterol, free and esterified cholesterol, triglycerides and free fatty acids along with total lipids and phospholipids in both intima and media of human and monkey thoracic aorta. Total lipids in intima were greater than that in media of both species. The higher value of intimal total lipids in human thoracic aorta was mainly due to total, free and esterified cholesterol. Phospholipids of intima and media of human thoracic aorta did not show variation, but relative percentage of phospholipids to total lipids in media accounted for 42% as against 30% in intima. There was no significant difference in triglycerides and free fatty acids in intima and media of human thoracic aorta. In monkey thoracic aorta, the higher value of intimal total lipids as compared to that of media was due to increase in all fractions of lipids. A comparison of human and monkey thoracic intima and media aortic lipids revealed a significantly lower value of free and esterified cholesterol in monkey aorta.

The values of phospholipid fractions of human and monkey thoracic aorta are given in table 2. There was no significant difference in total phospholipids as well as phospholipid fractions in intima and media of human thoracic aorta. But in monkey thoracic aorta, total phospholipids were significantly less in media as

Table 1. Neutral lipids and phospholipids of human and monkey thoracic aorta.

	Human[a]		Monkey[b]	
	Intima (mg/100 gm wet tissue)	Media	Intima (mg/100 gm wet tissue)	Meida
Total lipids	1502±280	1109±271***	1381±202	923±123***
Phospholipids	496±122	476±109	580±178	461±152*
Total cholesterol	660±137	354±121***	346± 42	175± 45***
Free cholesterol	363±102	194± 59***	190±30.5	91± 22***
Esterified cholesterol	297±110	159± 58***	155±27.4	80± 20***
Triglycerides	301± 80	233± 89	372± 37	239± 84***
Free fatty acids	75± 19	66± 14	103± 24	64± 18***

Values are Mean ±S.E.

[a] n—11, [b] n—18

* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Table 2. Human and monkey thoracic aorta phospholipid fractions.

	Human[a]		Monkey[b]	
	Intima (mg/100 g wet tissue)	Media	Intima (mg/100 g wet tissue)	Media
Total phospholipids	495±122	476±109	579±178	461±152
Phosphatidyl choline	121± 33	124± 27	163± 41	150± 32
Phosphatidyl ethanolamine	107± 24	102± 22	80± 30	72± 27
Sphingomyelin	132± 35	120± 27	170± 47	147±24.8
Lysophosphatidyl ethanolamine	27± 4.8	25± 4.2	30± 5.8	21± 5.2
Phosphatidyl serine	42±14.7	38± 9.7	38± 9.2	30± 5.8
Lysophosphatydylcholine	38±10.3	34± 7.8	36± 8.2	30± 7.9

Values are Mean ±S.E.

* $P<0.05$, [a] n—11, [b] n—18.

compared to that of intima. The different fractions of phospholipids remained unaltered in intima and media. If we compare human and monkey thoracic aortal phospholipid fractions, there was significant increase in phosphatidylcholine and sphingomyelin in monkey thoracic aorta. However, a significantly lower value of phosphatidyl ethanolamine was found in monkey thoracic aorta as compared to that of human thoracic aorta.

So far as abdominal aorta is concerned a significant increase in total lipids of intima as compared to that of media was noted in both monkey and man (table 3) which was contributed by total cholesterol and triglycerides. If we compare the lipid profile of human and monkey abdominal aortic intima and media, it is observed that there is significantly lower value of total, free and esterified cholesterol in both intima and media of monkey as compared to the corresponding region of human abdominal aorta.

Table 3. Neutral lipids and phospholipids of human and monkey abdominal aorta.

	Human[a]		Monkey[b]	
	Intima (mg/100 g wet tissue)	Media	Intima (mg/100 g wet tissue)	Media
Total lipids	1630±292	1237±201***	1491±254	1123±232***
Phospholipds	538±118	525±114	611±158	527±123^{NS}
Total cholesterol	717±130	383±109***	399± 97	235± 48***
Free cholesterol	394± 93	214± 81***	219± 82	129± 32***
Esterified cholesterol	322±103	166± 75***	175± 70	101± 27
Triglycerides	326±108	235± 72*	372±102	292± 81*
Free fatty acid	81± 23	68± 21	101± 22	78± 23**

Values are Mean ±S.E.

* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

[a] $n=11$, [b] $n=18$.

Table 4 contains the values of phospholipid content of abdominal aorta of monkey and man. A comparison of phospholipid content of human and monkey abdominal aorta revealed significantly higher content of phosphatidylcholine and sphingomyelin and with lower content of phosphatidyl ethanolamine in intima and media of monkeys as compared to that of human. Further, if we compare lipid constituents of human and monkey thoracic with abdominal aorta, the latter shows a higher lipid content in both species.

Discussion

From this study, certain similarities and dissimilarities in the lipid composition of human and monkey aorta have been elucidated. The present findings of lipid

Table 4. Human and monkey abdominal aorta phospholipid fractions.

	Human^a		Monkey^b	
	Intima	Media	Intima	Media
Total phospholipids	538±118	525±114	611±158	530±123
Phosphatidyl-choline	131± 32	124± 29	174± 40	159± 38
Phosphatidyl ethanolamine	115± 28	118± 34	75± 24	81± 24
Sphingomyelin	129± 32	112± 24	160± 38	140± 27
Lysophosphatidyl ethanolamine	49± 4.3	47±11.2	36± 8.9	31± 8.2
Phosphatidyl-serine	44± 7.8	49± 9.3	43±17.2	37± 5.8
Phosphatidyl-inositol	16± 4.3	12± 4.2	17± 5.6	13± 3.2
Lysophosphatidyl choline	41± 8.9	43±10.2	50±10.2	40± 9.8

Values are Mean ±S.E.

^{a} n—11, ^{b} n—18.

profile of intima and media of thoracic and abdominal aorta from human subjects are in conformity with previous reports (Buck and Bossiter, 1951; Smith, 1960; Bottcher *et al.*, 1960; Insull and Bartsch, 1966). There is no report in literature regarding the lipid profile of different layers and segments of monkey aorta. The neutral lipids were present in greater amount in intima of both thoracic and abdominal aorta as compared to the media in both man and monkey. This comparison supports the validity of Anitschkow's hypothesis (Anitschkow, 1925) that intima is freely permeable to the lipids of plasma and the intima wall exerts no selective action on these lipids. No significant difference could be observed in phospholipids of intima and media in both thoracic and abdominal aorta of both these species. However, relative percentage of phospholipids to total lipids, revealed higher level of this in media as compared to intima in both species, which could be the result of increased local synthesis (Besterman and Gillet,1971). There was more lipid in abdominal aorta as compared to thoracic aorta in both species, and this fact may explain the higher incidence of atherosclerosis in abdominal aorta (McGill *et al.*, 1964; Geer and Malcon, 1965). A comparison of lipid constituents of intima and media of thoracic and abdominal aorta of man with that of monkey revealed significantly lower value of free and esterified cholesterol in monkey aorta. This may be one of the factors which may explain lower incidence and extent of spontaneously occurring atherosclerosis in monkey (27%) (Chakravarti and Kukreja, 1981) as compared to man (80-90%).

Phospholipid fractions of human and monkey aorta revealed significantly higher level of phosphatidyl-choline and sphingomyelin in monkey thoracic and abdominal aortic intima and media as compared to corresponding areas in human aorta. As phosphatidylcholine is known to stabilize the hydrophobic lipids in solution and prevents its deposition in tissues and that it has a strong lipotropic action, atheroma formation can be effectively inhibited. The level of phosphatidyl ethanolamine was significantly decreased in intima and media of both thoracic and abdominal segments of monkey aorta as compared to that of human. The higher value of phosphatidyl ethanolamine in human aorta appears to be the result of increased vascular wall lipogenesis which may predispose the vessel to intravascular mural thrombosis, since phosphatidyl ethanolamine is known to promote platelet aggregation and fibrin formation (Turner *et al.*, 1963).

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Differential contractile response of normal vas deferens of rodents in correlation to their calcium and electrolyte levels*

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Abstract. The contractile pattern of the vas deferens in three different rodents, rat, guinea pig and mouse was studied in response to adrenaline and noradrenaline. The left vas deferens of rat was more responsive to the graded doses of adrenaline and noradrenaline than the right. The same was also true for guinea pig and mouse vas deferens. This differential response has been correlated with the greater concentrations of calcium and sodium in the right vas deferens in rats and guinea pigs and it might also be related to the levels of membrane-bound and intracellular calmodulin-bound calcium. It is suggested that the left vas deferens might possess more calmodulin-bound calcium than the right, which might have instead, more membrane-bound calcium.

Keywords. Vas deferens; calcium; contractile pattern.

Introduction

The interplay of metal ions on cellular functions and membrane structure are of importance in the reproductive system. The importance of calcium in the contractility of guinea pig vas deferens has been reported (Momose and Gomi, 1978; Sugimoto *et al.*, 1978). Moreover, the response of vas deferens to KCl also involves the participation of readily available calcium ions contained in the tissue (Sugimoto and Nagata, 1973; Sugimoto and Furumichi, 1975; Sugimoto *et al.*, 1977; 1978). Westfall *et al.* (1975) reported that the decrease in membrane-bound calcium ions leads to depolarization of the cell membrane and renders the cells more excitable. Recent studies by Slater (1981) have revealed that calcium is essential for norepinephrine release, but calcium alone cannot induce the release of neurotransmitters.

Although, it is known that continuous transport of the spermatozoa takes place due to regular spontaneous contractions (Hamilton, 1972), not only during ejaculation but also at rest, the mechanism of sperm transport through the ductus deferens is still not fully understood.

A study in three different rodents, rat, guinea pig and mouse, was carried out to investigate the effects of different doses of epinephrine and norepinephrine on the contractile pattern of their left and right vas deferens as well as the spontaneous contractions. The concentrations of Ca^{2+} and electrolytes Na^+, K^+ were especially

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Abbreviation used: NA, noradrenaline

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determined in order to correlate the changes, if any, in the contractile pattern of vas deferens, with the electrolyte profile, in the light of recent data on the role of calcium in smooth muscle contraction.

Materials and methods

Normal, mature, male, albino rats *(Rattus norvegicus)* or Holtzman strain (200-250 g body weight) adult healthy guinea pigs *(Cavia porcellus)* weighing between 400-450 g and mature albino mouse *(Mus musculus)* (40-50 g weight) were used for the study. All the animals used were of proven fertility. The animals were housed in a controlled environment at a constant temperature of 26 ± 2°C and were subjected to 12-14 h daylight. They were maintained on standard diets (Hindustan Lever Ltd., Bombay) and water was provided *ad libitum.*

Recordings of the isolated vas deferens

The recordings of the contractile pattern of the isolated vas deferens *in toto,* from normal adult rats, guinea pigs and mice were carried out using a standard technique as described earlier (Chinoy and Chinoy, 1979; 1981). These recordings were carried out simultaneously on the vas deferens from left and right sides of these rodents using a double organ bath and under identical conditions. The frontal lever was adjusted at 500 mg tension for rat vas deferens, 1000 mg for guinea pig and 75 mg for mouse vas deferens. The response to different doses of adrenaline from 1 to 500 µg was recorded in all rodents, whereas, the response to noradrenaline was recorded in the rat only.

The mean amplitude (in mm) of the individual response to each dose of adrenaline or noradrenaline in normal animals were calculated for the left and right vas deferens.

Atomic absorption spectrophotometry

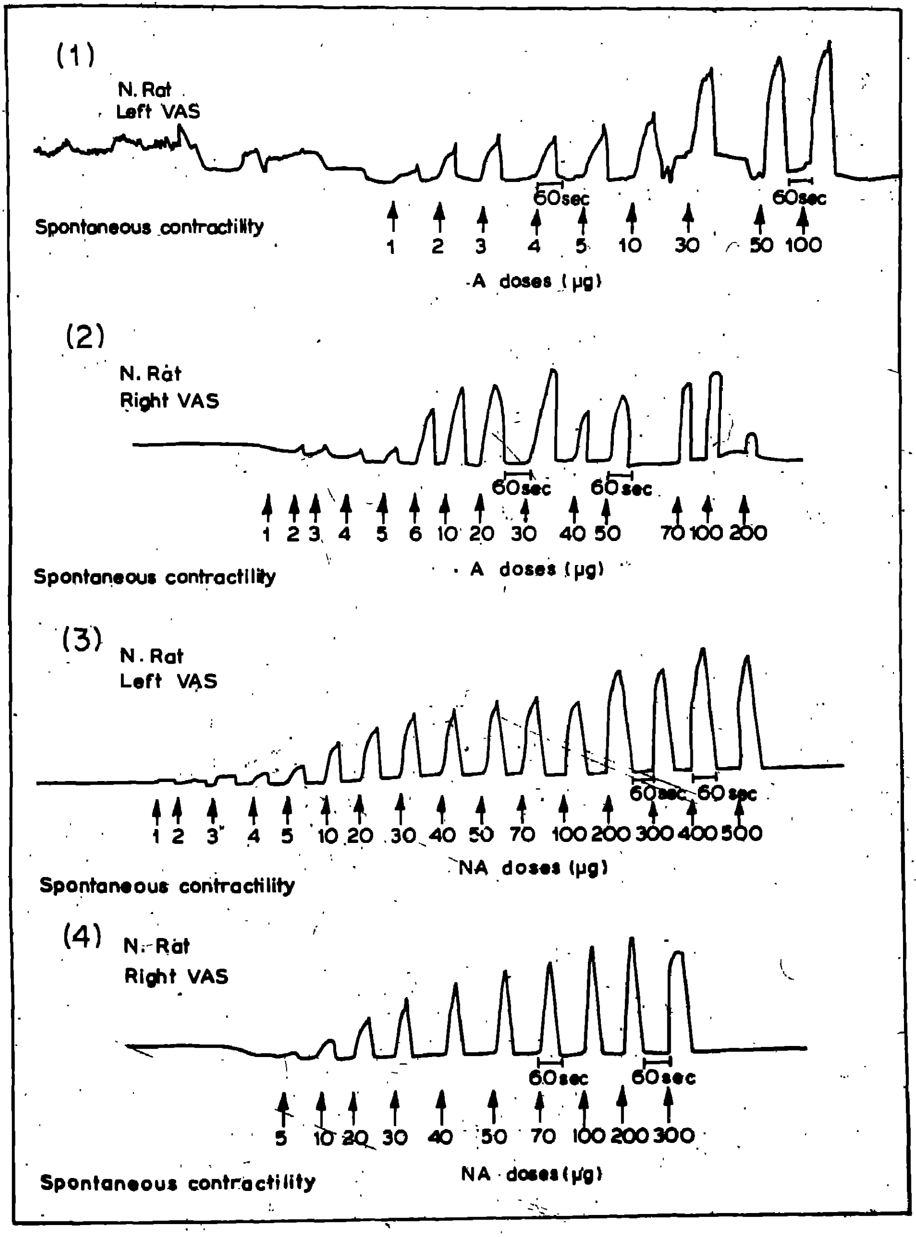
For the analysis of the different electrolytes in the vas deferens *in toto,* the study was carried out using the standard acid digestion method. Vas deferens from the left and right sides of the animals were processed separately. The concentrations of sodium, potassium and calcium were estimated separately using a Perkin Elmer 305 A atomic absorption spectrophotometer by the standard method.

For all analyses, a minimum of six replicates were done and the results were analyzed statistically by using the student's 't' test.

Results

Contractile pattern of the isolated vas deferens

Normal rat: The normal rat vas deferens from the left and right sides of the animal showed differences in the contractile pattern. Although, spontaneous contractility was obtained for the left, it was absent in the right vas deferens (figures 1, 2). It was also observed that the left vas deferens on the whole was more responsive to different doses of adrenaline than the right vas deferens. Moreover, the latter responded poorly to 200 µg of adrenaline, whereas, the left side did not respond at all. On both the sides, maximum amplitude was obtained with 100 µg adrenaline.



Figures 1-4. Recordings of the isolated vas deferens (left and right sides) of normal rat showing adrenaline (A) and noradrenaline (NA) induced contractions. **1.** Note the characteristic spontaneous contractile pattern of the left vas deferens as well as its response to different doses of A. **2.** Dose dependent contractile response of the isolated right vas deferens to A. Note absence of spontaneous contractions. **3.** No spontaneous contractility of the left vas and the response to different doses of NA was more uniform and higher than that by A. **4.** No spontaneous contractility of the right vas. It did not respond to 1 to 4 μg doses of NA. At higher doses it was less responsive as compared to the left vas.

The left vas deferens responded more to the different doses of noradrenaline (NA) than the right side. This holds good only for doses upto 100 μg NA, since beyond 100 μg dose, an irregularity in the contractile response was noted (figures 3, 4). The right vas deferens did not respond at all to lower doses of NA (1 to 4 μg). With 100 μg NA both the sides responded almost equally. In left vas deferens the highest amplitude was noted by 400 μg NA, whereas, in the right, the greatest response was with 200 μg dose of NA.

On the whole, a better response to exogenously applied NA at higher doses was elicited, whereas, at lower doses, adrenaline proved to be a more potent stimulant for vas deferens.

Normal guinea pig: The guinea pig ductus deferens from both the sides did not show spontaneous contractility. The left and right vas deferens were not responsive to 1 to 5 μg adrenaline, but a graded response was obtained with increase in dose of adrenaline and the amplitude was maximum with 400 μg adrenaline in the left as well as right. On the whole, left vas deferens was more responsive to all doses of adrenaline as compared to the right one, similar to the case in rat (figures 5, 6).

Normal mouse: The mouse also responded in a similar manner as the previous two rodents. No spontaneous contractions were observed in the vas deferens from left or right sides. Left vas deferens showed higher amplitude all throughout as compared to the right one (figures 7, 8).

On the whole, rat vas deferens from both the sides showed greater dose dependent response to 1 to 100 μg adrenaline, followed by mouse, and least by guinea pig. But guinea pig ductus deferens responded to the highest dose of adrenaline (400 μg) followed by rat (100 μg), and least by mouse (70 μg).

See, figure 9 (a, b, c, d) for comparative study of the contractile pattern of vas deferens in these rodents.

Metal ions

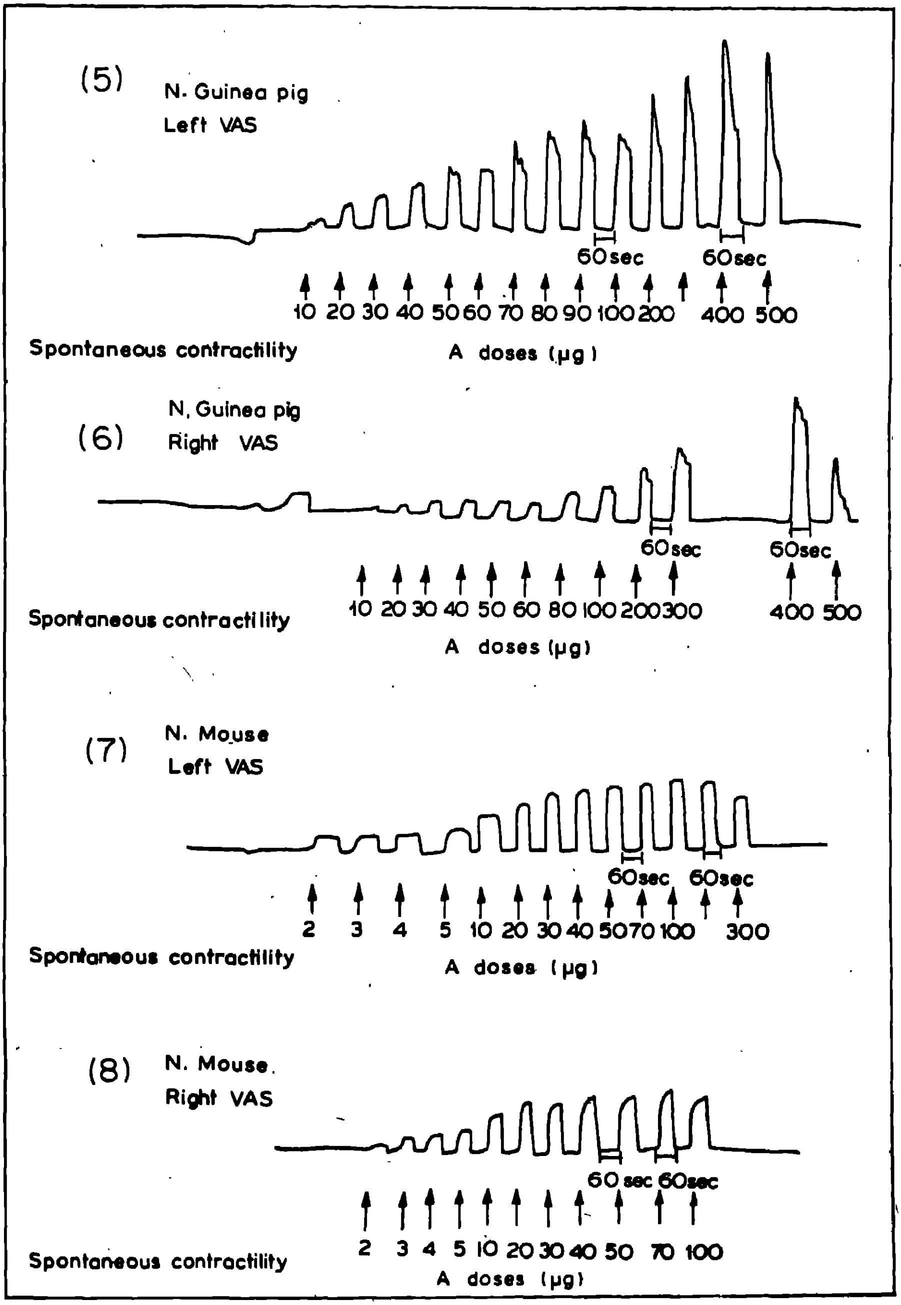
Sodium: In all the three animals, the Na^+ levels were found to be higher in the right vas deferens, than in the left one. This difference was significant in the mouse ($P<0.01$) and the rat ($P<0.02$), whereas in the guinea pig ductus deferens the difference was not significant ($P<0.1$) (table 1).

Potassium: Guinea pig left and right vas deferens possessed the highest concentrations of K^+ and both sides had more or less same levels. The mouse had lesser levels of K^+ than the guinea pig; the rat had the least especially in the right side ($P<0.05$) (table 1).

Calcium: Ca^{2+} concentrations were not detectable in left vas deferens of the rat and the guinea pig but were detectable in the right vas deferens. The levels were insignificantly higher in the guinea pig than in the rat. In the mouse vas deferens, calcium was not detectable at all (table 1).

Discussion

The vas deferens is not spontaneously active, and normally contracts only in response to neural stimulation. In the present study, spontaneous contractions



Figures 5-8. Recordings of the isolated vas deferens (left and right sides) of normal guinea pig and mouse, respectively, showing adrenaline (A) induced contractions. **5.** Note the absence of spontaneous contractility of the left vas in guinea pig. No response to lower doses of A (1 to 5 µg), but a graded response to the higher doses was noted. **6.** The right vas showed similar pattern of response in guinea pig, which was of significantly low amplitude. **7.** No spontaneous contractions and no response of the mouse left vas to 1 µg A, but a dose-dependent response to all higher doses was noted. **8.** The contractility of the right vas was similar to that of the left one, but response were of markedly low amplitude.

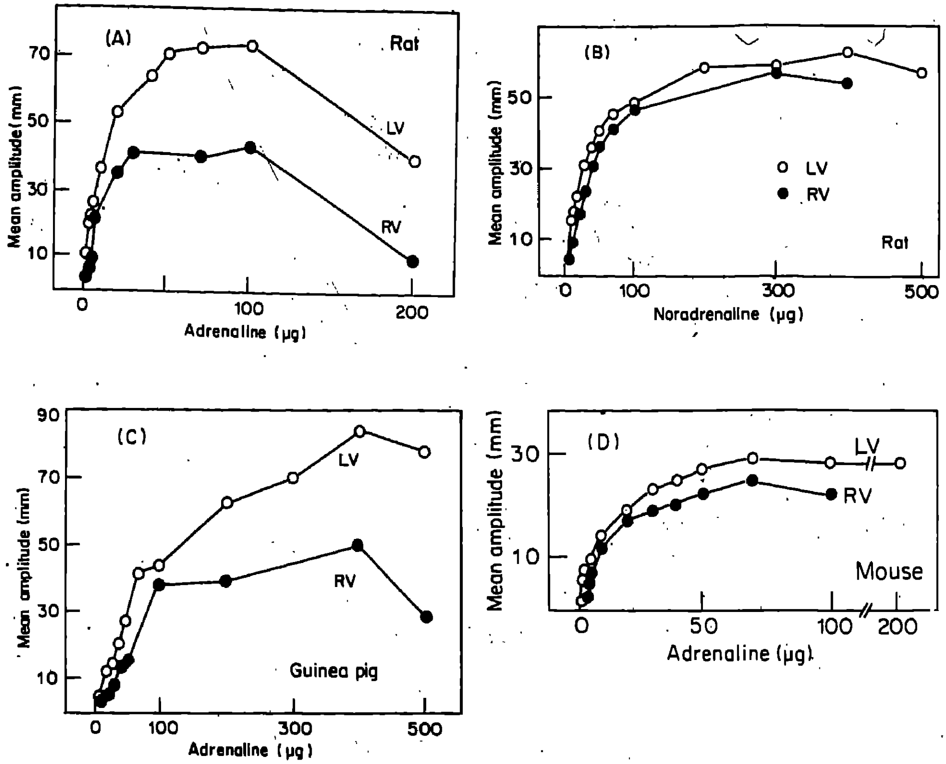


Figure 9. The graphs representing the mean amplitude of the induced response of the left and right vas deferens of rat, guinea pig and mouse plotted against the graded doses of adrenaline/noradrenaline.

LV—Left vas deferens; RV—Right vas deferens.

Table 1. Comparative data on the concentrations of different electrolytes and calcium (Na^+, K^+, Ca^{2+}) in the vas deferens from left and right sides of normal adult rat, guinea pig and mouse.

Experimental animal	Sodium* L.V.	Sodium* R.V.	Potassium* L.V.	Potassium* R.V.	Calcium* L.V.	Calcium* R.V.
Rat	1193.3 ±141.6	2576.7 ±587.8	3383.3 ±310.2	2655.0 ±282.9	N.D.	1404.4 ±370.5
Guinea pig	3595.7 ±794.7	4204.2 ±754.9	5104.2 ±247.5	5287.4 ±321.3	N.D.	1539.7 ±877.9
Mouse	2693.7 ±851.5	5152.03 ±1123.3	4366.2 ±1121.6	3523.3 ±858.5	N.D.	N.D.

Values are mean ±S.E. L.V.—Left vas deferens R.V.—Right vas deferens.
* μg/gm fresh tissue weights. N.D.—Not detectable.

were observed only in the left vas deferens of the rats. The nervous stimulation gives rise to excitatory junction potential which add to each other and when a critical value is reached, a spike is initiated. In the guinea pig, the spikes are all-or-none with overshoots of 20 mV. In the mouse, however, the spikes are graded in amplitude, varying from "humps" to overshoots of 5 mV (Bülbring and Bolton, 1979).

It is known that the density of innervation, and hence the effect of nerve activity shows much variation in different animals (Bülbring and Bolton, 1979), and that norepinephrine is the major neurotransmitter in rat, guinea pig and human vas deferens (Hafez, 1980); which also showed variations in its content in the human proximal and distal vas deferens (Baumgarten *et al.*, 1971). The density of innervation can therefore complement the physiological properties. In the present study too, the left vas deferens of rat was more responsive to different doses of adrenaline and noradrenaline (except at lower doses) than the right one. The same trend was observed for guinea pig and mouse left and right vas deferens.

Smooth muscle contraction is generally associated with alpha receptors and relaxation with β-receptors. The alpha receptor activation involves the increase in membrane permeability and calcium, whereas, the activation of β-receptor promotes the decrease of calcium and hence relaxation (Bülbring, 1979). This calcium which activates norepinephrine-induced contraction, originates from the loosely-bound calcium pool in extracellular fluid as well as from the pool of tightly-bound Ca^{2+} found intracellularly in the muscle fibre.

In the present study, the differential response of the vas deferens of the two sides from the same animal to adrenaline has been correlated with the greater concentrations of calcium and sodium in the right vas deferens of rats and guinea pigs, since it is known that the high levels of membrane-bound calcium renders the cells less excitable (Westfall *et al.*, 1975). The differential response of the left and right vas deferens might also be related to the levels of intracellular calcium, which is bound to calmodulin, a polypeptide (Cheung, 1960; Slater, 1981), which acts as an activator for norepinephrine release and muscle contraction as well as other cellular processes, only when bound to calcium. It is therefore probable that the left vas deferens might possess more calmodulin-bound calcium than the right, which has instead, more membrane-bound calcium. Hence, it is necessary to investigate further, the levels of bound and free forms of calcium in the vas deferens from the two sides, since it plays a key role in the release of neurotransmitters and muscle contraction via activation of calmodulin – the calcium binding protein (Lenz and Cormier, 1982). This calmodulin-bound calcium acting synergistically or antagonistically with c-AMP (Means *et al.*, 1982) is involved in discrete cellular activities as muscle contraction, neurotransmitter release, sperm motility etc.

The alpha-adrenergic nerves of the vas deferens help in transport of sperms and ejaculation (Bruschini *et al.*, 1977; Anton *et al.*, 1977). The exact mechanism of sperm transport needs further elucidation. Understanding of fine structure of smooth muscle of vas deferens as well as determination of levels of free and bound calcium and calmodulin would help to understand the mechanism whereby contractile pattern is modulated. The use of specific Ca^{2+} inhibitors or calmodulin binding drugs among them might be advantageous in the development of male contraceptives.

Acknowledgements

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Naphthalene oxygenase from *Corynebacterium renale:* Characterisation and mechanism of oxygenation

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Volume No. 5, Number 1

Page 10, legend to figure 1, sentence 2 should read as:

......with 2 μmol 1,10-phenanthroline in a total volume of 2.5 ml for 30 min at 30°C.....

Page 18, line 7 should read as:

groups of *cis*-1,2-dihydrodiol arise from H_2O_2......

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Evolutionary relationship of alfalfa mosaic virus with cucumber mosaic virus and brome mosaic virus

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MS received 5 August 1983

Abstract. The amino acid sequences of the non-structural protein (molecular weight 35,000; 3a protein) from three plant viruses—cucumber mosaic, brome mosaic and alfalfa mosaic have been systematically compared using the partial genomic sequences for these three viruses already available. The 3a protein of cucumber mosaic virus has an amino acid sequence homology of 33.7% with the corresponding protein of brome mosaic virus. A similar protein from alfalfa mosaic virus has a homology of 18.2% and 14.2% with the protein from brome mosaic virus and cucumber mosaic virus, respectively. These results suggest that the three plant viruses are evolutionarily related, although, the evolutionary distance between alfalfa mosaic virus and cucumber mosaic virus or brome mosaic virus is much larger than the corresponding distance between the latter two viruses.

Keywords. Brome mosaic virus; cucumber mosaic virus; alfalfa mosaic virus; sequence homology; evolutionary relationship.

Introduction

Plant viruses are grouped into different classes based on a detailed examination of their host and vector specificity, serological relationships, particle morphology, nucleic acid content and nature, and genomic organization. Alfalfa mosaic virus (AMV), cucumber mosaic virus (CMV) and brome mosaic virus (BMV) are classified into different families of plant viruses, (Matthews, 1979) although they have a similar genomic organization. AMV (Jaspars, 1974), CMV (Kaper and Waterworth, 1981) and BMV (Lane, 1981) are single stranded, positive sense RNA viruses with functionally divided genomes. CMV and BMV are icosahedral viruses based on a $T=3$ lattice (for definition, see Caspar and Klug, 1962), while AMV particles are bacilliform, probably with $T=1$ ends (Driedonks *et al.*, 1977). The coat proteins of these viruses are serologically unrelated. They also differ widely in their host and vector specificities. Despite these differences, they exhibit a remarkable similarity of genomic organization. Purified RNA from all the three viruses consist of four RNA segments with approximate molecular weights of 1.3, 1.0, 0.7 and 0.3 million daltons, respectively. RNA1, RNA2 and RNA4 are monocistronic. RNA4 is the messenger for the coat protein. RNA3 is dicistronic and codes for a non-structural protein; M_r 35,000 called 3a protein of unknown

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Abbreviations used: AMV, Alfalfa mosaic virus; CMV, cucumber mosaic virus; BMV, brome mosaic virus; TSV, tobacco streak virus.

function and the coat protein. Apart from these similarities in genomic organization, these viruses have many common physical and chemical properties, which has led Kaper to group these viruses together (Kaper, 1975). Hence, it would be of interest to know if an evolutionary relationship exists between these viruses.

Recently, it has been shown that CMV is evolutionarily related to BMV (Murthy, 1983). In the present study, the amino acid sequence of the non-structural protein of AMV (M_r 35,000; 3a protein)was compared with the sequences of the corresponding proteins of CMV and BMV in an attempt to determine their evolutionary relatedness. The results reveal a significant homology between AMV and CMV or BMV with an evolutionary distance much larger than the corresponding distance between the latter two viruses.

Materials and methods

Complete nucleotide sequence of RNA3 and hence the sequences of the coat protein and the 3a protein of these viruses are now available (Barker *et al.*, 1983; Gould and Symons, 1983; Ahlquist *et al.*, 1981). The 3a proteins of AMV, BMV and CMV are 299, 303 and 333 amino acids in length, respectively. In an earlier study, the 3a protein sequences of BMV and CMV were shown to possess a homology of 33.7% (Murthy, 1983). In the present study, the 3a protein sequence of AMV was compared to the corresponding sequences of BMV and CMV by the method of Jukes and Cantor (1969). In this method, segments of length *l* from the first amino acid sequence are systematically compared to all possible segments of the same length in the second molecule and the homology between them is evaluated. The segment length chosen for comparison was 20 residues. The observed frequency of segments with a high degree of homology was not significantly greater than the expected frequency for unrelated sequences. This could be the case if the sequences are either unrelated or distantly related. However, six segments with good homology (30%) between AMV and BMV or CMV proteins could be identified at AMV residues 1–20, 48–67, 105–124, 157–176, 208–227, 274–293. Accepting these regions as equivalent, the other regions were manually adjusted so as to achieve overlap of residues with high relative substitution frequencies (McLachlan, 1971).

Results and discussion

A total of 264 positions were found to be common for all the 3 sequences. Identical residues at 70 of these positions were found between AMV and either CMV or BMV 3a proteins. This corresponds to a homology of 26.5%. Identical residues were found in all the three sequences at 20 of these positions. The minimum base change per codon (MBC/C) required to convert the AMV 3a protein sequence to the sequence obtained by selecting suitable residues from corresponding positions of BMV or CMV was 0.75. In pairwise comparisons, 281 residues were common between AMV and BMV. 51 of these were found to be identical (18.2%). The MBC/C value for these 281 positions was 1.31. With CMV, 274 common and 39 identical (14.2%) residues were found. The MBC/C value for the 274 positions was 1.31. The final alignments are shown in table 1. 48.0% of the residues are

Table 1. Alignment of the 3a protein sequences of cucumber mosaic, brome mosaic and alfalfa mosaic viruses.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
CMV	-	-	-	-	-	M	A	F	P	G	P	S	R	T	L	T	Q	Q	S	S
BMV	M	S	N	I	V	S	P	F	S	G	S	S	R	T	T	S	D	V	G	K
AMV	M	E	N	T	K	T	N	A	S	S	S	G	M	S	S	S	S	S	F	S
CMV	A	A	L	P	D	D	-	-	-	L	Q	K	I	L	F	S	P	D	A	I
BMV	Q	A	G	G	T	S	D	E	K	L	I	E	S	L	F	S	E	K	A	V
AMV	V	S	Y	A	E	E	M	L	L	A	D	E	V	S	K	I	N	S	M	S
CMV	K	K	M	A	T	E	C	D	L	G	R	H	H	W	M	R	A	D	N	A
BMV	K	E	I	A	A	E	C	K	L	G	C	Y	N	Y	L	K	S	N	E	P
AMV	I	L	G	P	N	Q	L	K	L	C	T	Q	L	V	L	S	N	G	A	A
CMV	I	S	V	R	P	L	V	P	Q	V	T	S	N	N	L	L	S	F	F	K
BMV	R	N	Y	I	D	L	V	P	K	S	H	V	S	A	W	L	S	W	A	T
AMV	P	V	V	L	S	L	V	S	K	E	K	K	S	I	L	N	R	M	L	-
CMV	S	G	Y	D	A	G	E	L	R	S	K	G	Y	M	S	V	P	Q	V	L
BMV	S	K	Y	D	K	G	E	L	P	S	R	G	F	M	N	V	P	R	I	V
AMV	-	-	-	-	-	P	K	I	G	Q	R	M	Y	V	H	H	S	A	I	Y
CMV	C	V	V	T	R	T	G	S	T	D	A	E	G	S	L	K	I	Y	L	A
BMV	C	F	L	V	R	T	T	D	S	A	E	S	G	S	I	T	V	S	L	C
AMV	L	L	Y	M	P	N	I	L	K	S	S	S	G	S	I	T	L	K	L	F
CMV	D	L	G	D	K	E	-	-	-	-	L	S	P	I	D	G	Q	C	V	T
BMV	D	S	G	K	A	A	R	A	G	V	L	E	A	I	D	N	Q	E	A	T
AMV	N	E	A	T	G	E	L	V	D	V	D	T	D	H	D	A	T	Q	A	C
CMV	L	H	N	H	E	L	P	A	L	I	S	F	Q	P	T	Y	D	C	P	M
BMV	I	Q	L	S	A	L	P	A	L	I	A	L	T	P	S	Y	D	C	P	M
AMV	I	F	A	G	R	Y	P	R	S	I	L	A	K	D	A	A	K	G	H	D
CMV	E	-	-	L	V	G	-	-	-	N	R	H	R	C	F	A	V	V	V	E
BMV	E	-	-	V	I	G	G	D	S	G	R	N	R	C	F	G	I	A	T	Q
AMV	L	K	L	V	V	H	A	V	A	S	T	N	A	N	S	A	V	G	V	-

Table 1· *Contd.*

CMV	R	H	G	Y	I	G	Y	G	G	T	T	A	S	V	C	S	N	W	Q	A
BMV	L	S	G	V	V	G	T	T	G	S	V	A	V	T	H	A	Y	W	Q	A
AMV	L	Y	P	I	W	E	D	E	L	S	R	K	Q	I	L	E	R	-	G	A
CMV	Q	F	S	S	K	N	N	N	Y	T	-	-	-	-	-	H	A	A	A	G
BMV	N	F	K	A	K	P	N	N	Y	K	-	-	-	-	-	L	H	G	P	A
AMV	D	F	L	K	F	P	I	A	E	T	E	P	V	R	D	L	L	N	A	G
CMV	K	T	L	V	L	P	Y	N	R	L	A	E	H	S	K	P	S	A	V	A
BMV	T	I	M	V	M	P	F	D	R	L	R	Q	L	D	K	K	S	-	L	K
AMV	K	L	T	D	F	V	L	D	R	T	R	-	-	-	-	-	-	-	-	-
CMV	R	L	L	K	S	Q	L	N	N	V	S	S	S	R	Y	L	L	-	-	P
BMV	N	Y	I	R	G	I	S	N	Q	S	V	D	H	G	Y	L	L	G	R	P
AMV	-	-	-	L	G	V	G	S	K	N	D	P	S	P	V	L	L	E	-	P
CMV	N	V	A	L	N	Q	N	A	S	G	H	E	S	H	I	L	K	E	S	P
BMV	L	Q	S	V	D	Q	V	A	Q	E	D	L	L	V	E	E	S	E	S	P
AMV	R	A	K	I	T	G	K	A	K	T	V	F	I	P	E	G	P	S	V	P
CMV	P	I	A	I	G	S	L	S	A	S	R	N	N	S	F	R	S	Q	V	V
BMV	-	S	A	L	G	R	-	G	V	K	D	S	K	S	V	S	A	S	S	V
AMV	N	T	I	N	G	M	-	-	-	A	P	T	V	R	I	D	A	G	S	P
CMV	N	G	L	C	A	L	L	R	C	T	Y	V	-	Y	I	Y	Y	V	Y	L
BMV	A	G	L	P	V	S	S	P	T	L	R	I	K							
AMV	K	E	L	G	V	P	K	G	F	T	Y	E	S	F	I	K	D	E	I	L
CMV	P	Y	V	N	T	C	E	S	R	V	P	C	E	L	S	T	V	D	I	C
AMV	P	D	H																	
CMV		D	D	A	L	E	D	F	D	H	G	V	S	K	Y	T	S	Q	F	

The residues that are identical in at least two sequences are shown in boxes. Pairs of residues with high relative substitution frequencies are underlines. Single letter codes for amino acids (Schultz and Shirmer, 1979) have been used.

either identical or conservative substitutions (McLachlan, 1971) between AMV and BMV or CMV. The homology observed between these sequences is usually accepted as a definitive indication of an evolutionary relationship.

Van Tol and Vloten-Doting (1981) have observed lack of serological cross-reactivity between the 3a proteins of AMV and the corresponding proteins of CMV, BMV and tobacco streak virus (TSV). However, the same authors note that the 3a proteins might still be functionally equivalent since the coat protein of AMV activates TSV genome, although it does not show serological relationship with TSV coat protein.

In addition to the similarity of genomic organization, AMV, BMV and CMV exhibit similarity in their sensitivity towards sodium dodecyl sulphate, salt and ribonuclease. This has led Kaper (1975) to group them together as viruses stabilized mainly by protein-nucleic acid interactions. The results presented in this paper (table 1) suggest that AMV has indeed diverged from an ancestral precursor of BMV and CMV, and hence Kaper's classification scheme has an evolutionary basis. The x-ray crystallographic investigation of AMV (Fukuyama *et al.*, 1983) and cowpea chlorotic mottle virus (Rayment *et al.*, 1977) which is a bromo virus, are now in progress. A comparison of the structures of the coat protein of these two viruses, when they become available, should provide further understanding of the evolutionary relationship of these two viruses.

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Analysis of alphavirus polypeptides by two dimensional polyacrylamide gel electrophoresis

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Abstract. Semliki Forest, Sindbis and Chikungunya viruses were grown and radiolabeled with [^{3}H]-amino acids in Vero cells. Analysis of virus infected cell lysates by two dimensional polyacrylamide gel electrophoresis resulted in detection of polypeptides of molecular weights corresponding to those of E1, P62, ns60, ns70/72 for Semliki Forest virus, the C, E1, 6K, 14K, PE2, P97, ns60, ns82 for Sindbis virus and E1, P62, P97, ns70/72 for Chikungunya virus. Charge and molecular weight heterogeneity in the precursor polypeptide P62 of Semliki Forest virus was detected. Structural polypeptides e.g. E1 and E2 of Semliki Forest virus and C, E1, E2 of Sindbis virus and E1 of Chikungunya virus were detected when purified radiolabeled virus preparations were analyzed by two dimensional polyacrylamide gel electrophoresis. Membrane glycoprotein E1 and E2 of Semliki Forest and E1 of Sindbis and Chikungunya viruses exhibited charge heterogeneity. In contrast to the marked difference in isoelectric points of E1 and E2 of Sindbis virus; E1 and E2 of Semliki Forest virus had almost identical isoelectric points.

Keywords. Alphaviruses; membrane glycoproteins; charge heterogeneity; two dimensional protein gels.

Introduction

Alphaviruses such as Sindbis (SIN), Semliki Forest (SF) and Chikungunya (CHIK) are single stranded RNA viruses containing a lipid envelope made up of two or three spike glycoproteins. The envelope surrounds an icosahedral nucleocapsid consisting of capsid protein (C) and 49 S genomic RNA of positive polarity (Matthews, 1979). Owing to their relatively simple structure, these viruses are often used as models for the study of cellular membranes (Klenk and Rott, 1980; Simons *et al*, 1978). The presence of membrane glycoproteins is essential for the infectivity of the virions (Uterman and Simons, 1974). Other biological properties such as haemagglutination, complement fixation and induction of neutralizing antibodies have been assigned to them (Dalrymple *et al*, 1976; Hashimoto and Simizu, 1979; Helenius *et al*, 1976; Symmigton *et al*, 1977). These glycoproteins can also serve as epidemiological markers (Wiebe and Scherer, 1980). Post-translational modifications such as cleavage, (Klenk and Rott, 1980) glycosylation, (Leavitt *et al*, 1977; Klenk and Rott, 1980; Schlesinger *et al*, 1976) phosphorylation, (Waite *et al*, 1974) sulphation (Pinter and Compans, 1975) acylation, (Rice and Strauss, 1981; Schmidt, 1982; Schmidt *et al*, 1979), are known for these

Abbreviations used: SF, Semliki Forest; SIN, Sindbis; CHIK, Chikungunya viruses; WEE, Western equine encephalitis; 2D PAGE, 2 dimensional polyacrylamide gel electrophoresis; NTE buffer, 0.01 M Tris-HCl buffer pH 8.0 containing 0.1 M NaCl, 0.001 M EDTA; SDS, sodium dodecyl sulphate; DMSO, dimethyl sulphoxide.

membrane glycoproteins. Some of these post translational modifications are known to contribute to the electrical charges of proteins (O'Farrell, 1975). The charge heterogeneity in some of the structural polypeptides of negative strand RNA viruses have been detected by two dimensional polyacrylamide gel electrophoresis (2 D PAGE) (Rhaghow *et al*., 1978; Privalsky and Penhot, 1978; Leavitt *et al*., 1979). In the present study the polypeptides of SIN, CHIK and SF viruses were analysed by 2 D PAGE. Marked charge heterogeneity amongst the membrane glycoproteins was detected.

Materials and methods

Cell and viruses

Vero (African green monkey kidney) cells were grown in Eagle's minimal essential medium supplemented with 5% goat serum heat-inactivated at 56° C for 30 min and antibiotics (pencillin G 100 units/ml, streptomycin sulphate 100 μg/ml). SIN virus (strain AR339), SF virus (strain NIV 718488) and CHIK virus (strain NIV 634029) were employed in the study.

Preparation of labeled cell lysates

Vero cells were grown in plastic Petri-dishes (50 mm dia.) confluent monolayers containing $2-3\times10^6$ cells were infected at a m.o.i. of 3–5 Plaque forming units/cell. The virus was adsorbed on the cells at 37° C for 2 h following which the plates were washed, and fresh medium containing [^{3}H]-leucine, [^{3}H]-alanine and [^{3}H]-arginine (obtained from Bhabha Atomic Research Centre, Bombay), each at concentration of 5 μCi/ml, was added to the cultures. The cultures were pulsed at 37° C for 10 h. At the end of the incubation period the monolayers were lysed in sonication buffer (0.01 M Tris-HCl buffer, pH 7.4, 5 mM $MgCl_2$ DNase 50 μg/ml RNase 50 μg/ml). Samples containing acid precipitable 2×10^5 tritium cpm, were analysed by 2 D PAGE.

Preparation of purified labeled viruses

Vero cells were grown in 2 litre roller bottles, and infected as above but were labeled for 20 h at 37° C. Cultures were harvested by repeated cycles of freezing and thawing and the culture fluid was centrifuged at 3500 *g* for 30 min at 4° C. Concentration and semipurification of the virus in the supernatant fluid, was simultaneously achieved by sedimenting the virus through a 10 ml cushion of 30% glycerol in NTE buffer (0.1 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA pH 8.0) in a Beckman SW 27.5 rotor at 25,000 rpm for 90 min. The pelleted virus was reconstituted in 100 μl of NTE buffer and was loaded on a 5 ml of 10–35% preformed linear gradient of sucrose in NTE buffer and was centrifuged at 35,000 rpm in a Beckman SW 50.1 rotor for 60 min. Fractions were collected in 0.5 ml quantities; haemagglutination titres and trichloroacetic acid precipitable radioactivity in each fraction was determined. To the pooled positive fractions bovine serum albumin to a final concentration of 50 μg/ml was added as a carrier protein and precipitation was carried out by the addition of cold trichloroacetic acid by the method of Barbacid *et al.* (1980). The precipitate was washed with 3 cycles of ice-cold acetone before dissolving it in lysis buffer (9 M urea, 2% NP40, 2% ampholines, 5% β-mercaptoethanol). Samples containing acid precipitable 2×10^3 tritium cpm were analysed by 2 D PAGE.

2 D PAGE

The method devised by O'Farrell (1975) for 2 D PAGE was employed. Isoelectric focusing in first dimension was carried out in standard tube gel electrophoresis chamber. At the end of isoelectric focusing in first dimension, gels were equilibriated with sodium dodecyl sulphate sample buffer. A gel which did not receive any sample was processed for determination of pH gradient. Gels for second dimension were made up of either 10% acrylamide or 5–12.5% linear gradient of acrylamide. Vertical slab gel electrophoretic apparatus as described by Studier (1973) was employed. Electrophoretic conditions for 2 D PAGE were as described by O'Farrell (1975). Lysozyme (14 K), trypsinogen (24 K), ovalbumin (45 K) and bovine serum albumin (67 K) (obtained from Sigma Chemical Co., St. Louis, Missouri, USA) were included in the analysis run as molecular weight markers. At the end of the electrophoresis, gels were fixed in 50% trichloroacetic acid and then were stained with 0.1% Coomassie brilliant blue in 50% trichloroacetic acid and destained with 7% acetic acid.

Fluorography

Film detection method for tritium labeled proteins as described by Bonner and Laskey (1974) was employed in the study. Destained gels were soaked in dimethyl sulphoxide (DMSO) for 30 min followed by a second 30 min immersion in fresh DMSO. Gels were then immersed in 20% PPO (2–5 diphenyl oxazole) in DMSO for 3 h followed by overnight immersion in water. Gels were dried under vacuum for 1 h in a gel drier (Bio-Rad). Dried gels were then exposed to Kodak X-Omat R.P. films for 40 days at −60° C.

Results

The isoelectric focusing in gels generated linear pH gradient from 4.0 to 8.0 even though the ampholine employed was of the pH range 3 to 10. This was consistently observed in the present series of experiments, and also by O'Farrell (1975). The fluorographs from the infected cell lysates and the purified viruses were compared with the fluorographs of appropriate controls (figures 1A and 2A). The designation of alphaviral proteins used in this paper are after Kaariainen and Soderlund (1978). Of the SF virus specific proteins, E1, E2, P62, ns60 and ns72 were detected in 2 D PAGE (see figure 1B and table 1). The membrane glycoproteins E1 and E2 had identical isoelectric points. Both displayed charge heterogeneity. Both E1 and E2 resolved in two distinct subspecies of proteins (isoproteins) differing in charge (pI 7.6 and pI 7.8). Analysis of the purified SF virus by 2 D PAGE using 10% SDS acrylamide in the 2nd dimension, allowed clear separation of E1, from E2 with their subspecies with pI 7.4, 7.8 and pI 7.6, 7.8 respectively (figure 2B). Precursor protein P62 from SF infected cells also exhibited charge heterogeneity. This precursor protein (P62) exhibited as six subspecies with molecular weights ranging from 64 to 60 K and having pI 6.6, 6.8, 6.9, 7.2, 7.3 and 7.6. Gradual decrease in molecular weights from 64 K to 60 K was observed with concomitant increase in pI from 6.6 to 7.6 (figure 1B and table 1).

Though P62 could be resolved into six isoproteins the intensity of each of them in the fluorograph was not uniform. The density of the spot made by the isoprotein

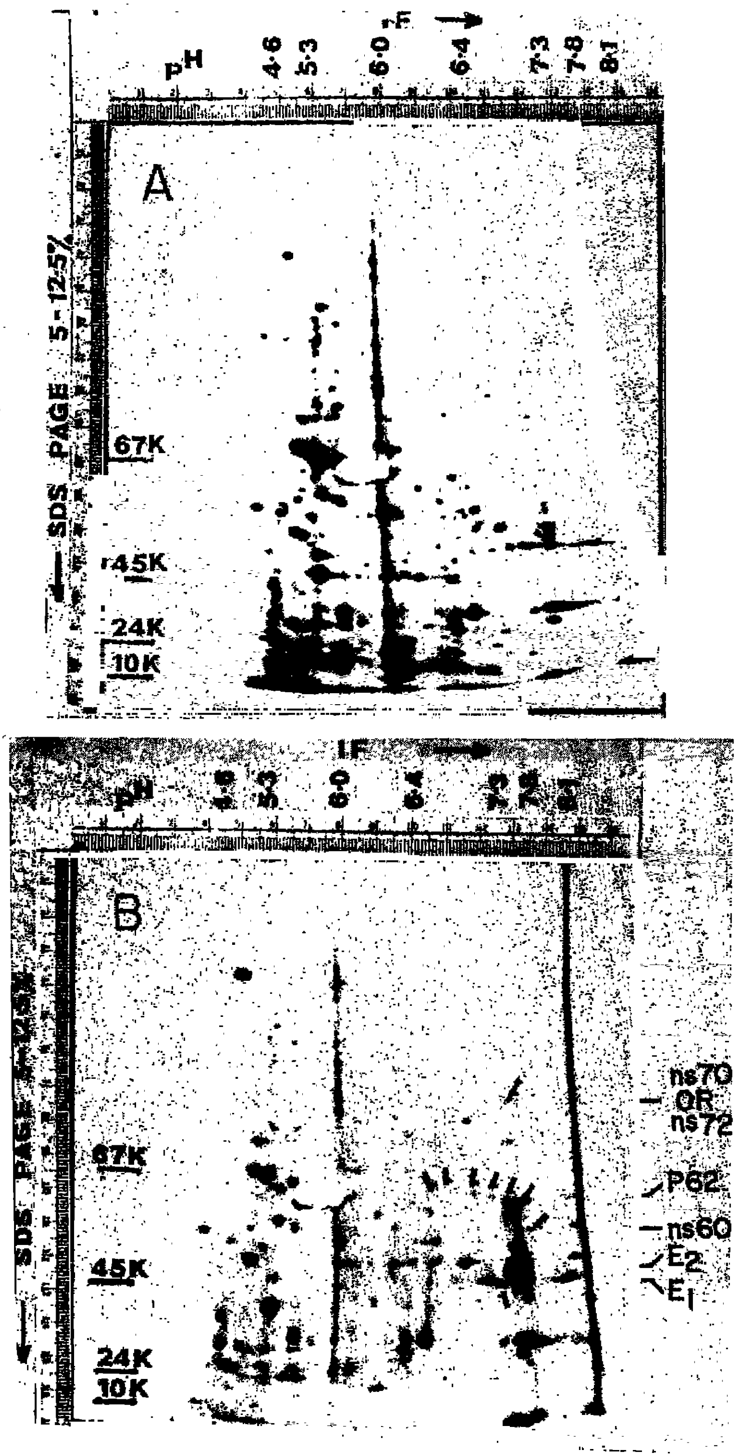

Figure 1. (*Captions in page 193*).

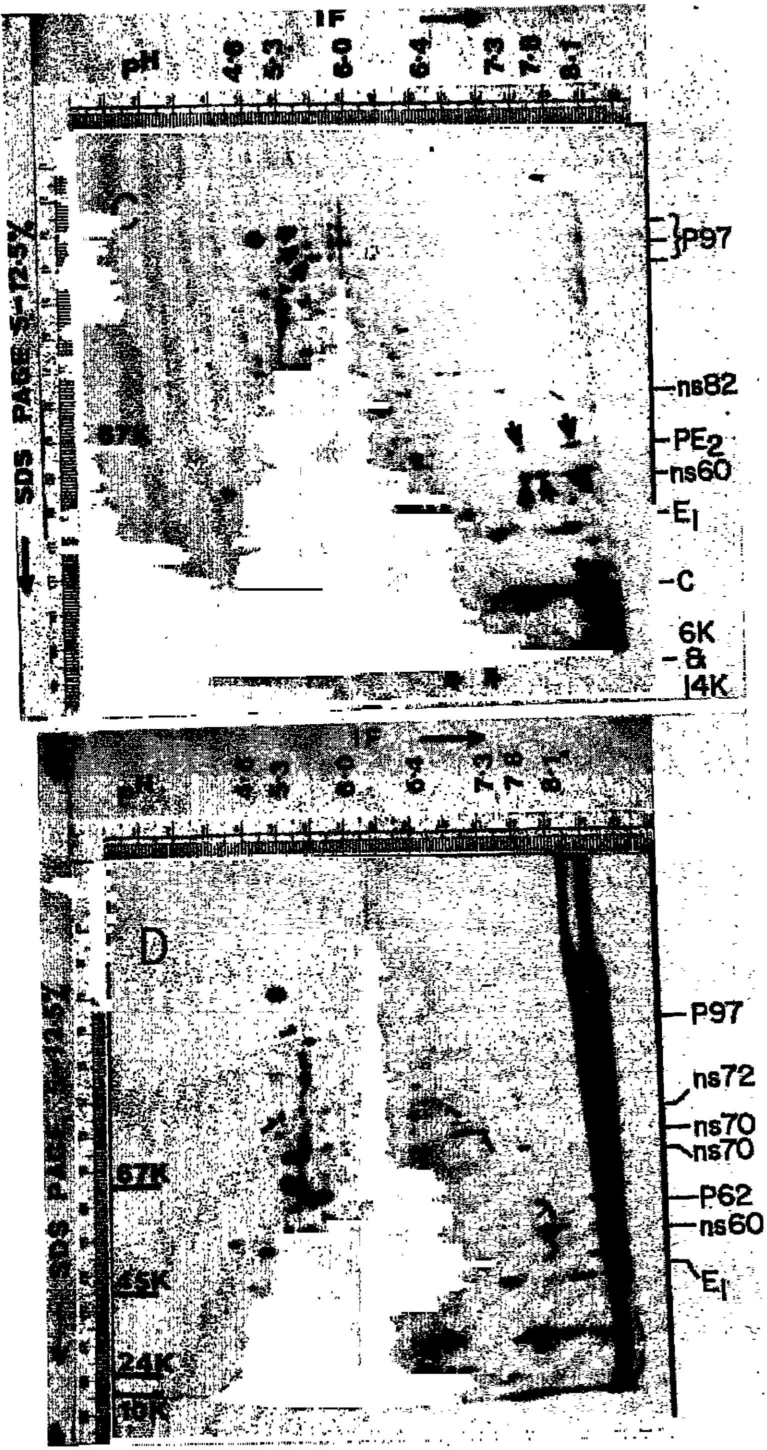

Figure 1. Two dimensional polyacrylamide gel electrophoresis of lysates from alphavirus infected cells. Vero cells were infected and pulse labeled with [^{3}H]-amino acids for 10 h; lysates were prepared and electrophoresed as described in the text. **A.** Mock infected. **B.** SF virus infected. **C.** SIN virus infected. **D.** CHIK virus infected. Arrows indicate viral polypeptides.

pI 7.6 (60 K) was highest. This protein appeared as a vertical streak suggesting that it contained molecules with variable molecular weights ranging from 60 to 57 K but having identical pI.

Another polypeptide, 57 K, (pI 7.85) was detected. It was of low density, the origin of which is conjectural. Possibly it is a representative of the nonstructural protein ns60.

Sindbis virus (SIN)

Cells infected with SIN virus when analysed by 2 D PAGE, showed viral specific polypeptides of molecular weights corresponding to those of P97, ns82, PE2, ns60, E1, C and 6 K (figure 1C and table 1). In purified virus preparation (figure 2C), only proteins of molecular weights corresponding to those of E1, E2 and C were detected. SIN virion membrane polypeptide E1 (50 K) displayed charge heterogeneity both in purified as well as in infected cell lysates. Three subspecies (isoproteins) of E1 from purified virus had pI 6.1, 6.2, 6.3 while another membrane protein E2 appeared as single homogeneous spot at pI 8.0. Another protein (30 K) corresponding to that of capsid protein was also detected (pI 8.1).

Chikungunya virus (CHIK)

CHIK virus infected cell lysates showed virus specific proteins of molecular weights corresponding to those of P97, ns80, ns70, P62/PE2, ns60 and E1, in 2 D PAGE (figure 1D and table 1). The membrane glycoprotein E1 displayed charge heterogeneity (pI 6.3, 6.5 and 6.7). In purified CHIK virus preparation (figure 2D) the E1 was the only polypeptide visible, other structural polypeptides E2 and C could not be detected. The polypeptide E1 from purified virus preparation also displayed charge heterogeneity similar to that found in cell lysates (figure 2D and table 2).

Discussion

In isoelectrofocusing pH gradient was obtained in the range of 4–8; therefore only those proteins having pI within this range could be detected efficiently. Many structural proteins such as E2 and C of CHIK, E3 and C of SF, and sometimes E2 of SIN virus were not detected. Aggregated or undissociated proteins as well as those having very high (basic) pI failed to enter the isoelectric focusing gel completely resulting in vertical streak in the SDS gel in the 2nd dimension. The proteins located in this type of streak (in the extreme right of the fluorographs) may have very high pI (greater than 8) or may represent the undissociated proteins failing to enter the IF gel (O'Farrell, 1975). For instance, the glycoprotein E2 of SIN virus located in such a streak (figure 2C); probably has a pI greater than 8.0. Thus our results indicating pI 6.1 to 6.5 for subspecies of E1 and pI 8.0 or more than 8.0 for E2 of SIN virus are in agreement with the earlier reported values i.e. pI 6.0 for E1 and pI 9.0 for E2 (Dalrymple *et al*, 1976). The capsid protein C of SIN virus also appeared in the streak; its pI was ≥8.0 which however differs from the value reported earlier (pI 4.0) by Dalrymple *et al* (1976). However, independent data from amino acid composition (Bell *et al*, 1979) and amino acids sequencing (Rice and Strauss, 1981) indicate enrichment of basic amino acids in the capsid protein

Table 1. Detected Polypeptides of Sindbis, Semliki Forest and Chikungunya viruses from infected cell, analysed by 2 D PAGE.

Proteins specified by alphaviruses their molecular weights and designations	Semliki Forest virus (SF)			Sindbis virus (SIN)			Chikungunya (CHIK)		
	Figure 1B			Figure 1C			Figure 1D		
	Mol. wt. K	Iso-electric points pI	Coordinates X and Y	Mol. wt. K	Iso-electric points pI	Coordinates X and Y	Mol. wt. K	Iso-electric points pI	Coordinates X and Y
Capsid (C) 30K		ND		35K	≥8.0	14.8×13.6		ND	
E1 50K	48K	7.6	12.8×12.2	52K	6.3	10.0×11.3	50K	6.3	8.8×12.1
Virion membrane glycoprotein	48K	7.8	13.2×12.2	52K	6.4	10.1×11.3	50K	6.5	9.2×12.1
				52K	6.6	10.6×11.3	50K	6.7	9.7×12.1
E2 50K	50K	7.6	12.8×11.8		ND			ND	
Virion membrane glycoprotein	50K	7.8	13.2×11.8						
6K/14K		ND		< 10K	6.5	10.5×15.5		ND	
				≤ 10K	7.2	10.0×15.5			
P62/PE2	60K	7.6	13.0×10.1	68K	7.75	12.9× 9.3	62K	8.0	12.4×10.3
Precursor to structural protein E2	62.5K	7.3	12.8× 9.9	68K	8.1	14.3× 9.3			
	63.5K	7.2	12.2× 9.7						
	64K	6.9	11.7× 9.5						
	64K	6.8	11.1× 9.5						
	64K	6.6	10.7× 9.5						
P97		ND		102K	5.7	6.8× 3.9	98K	5.45	5.5× 4.8
				104K	5.45	6.6× 3.5			
				107K	5.45	6.6× 3.0			
ns60	57K	7.85	13.6×10.6	64K	7.5	13.0×10.1	58K	8.0	12.6×11.1
				64K	7.8	13.4×10.1			
ns82		Not present in SFV		78K	6.1	8.3× 8.1		ND	
				78K	6.0	8.0× 8.0			
ns70/ns72	77K	7.5	12.8× 6.9		Not present in Sindbis		72.5K	7.2	10.8× 8.0
							74K	5.1	4.8× 7.5
							75K	6.6	9.5× 7.3
							70K	5.9	6.7× 8.6

ND—Not detected

Table 2. Detected structural polypeptides of Sindbis, Semliki Forest and Chikungunya viruses from purified preparations, analysed by 2 D PAGE.

Structural proteins specified by alphaviruses with their molecular weights and designation	Semliki Forest virus (SF)			Sindbis virus (SIN)			Chikungunya (CHIK)		
	Figure 2B			Figure 2C			Figure 2D		
	Mol. wt. K	Iso-electric points pI	Coordinates X and Y	Mol. wt. K	Iso-electric points pI	Coordinates X and Y	Mol. wt. K	Iso-electric points pI	Coordinates X and Y
C 30K Capsid protein		ND		32K	≥8.0	15.2×6.0		ND	
E1 50K	48K	7.4	11.8×8.8	50K	6.1	9.8×3.8	53K	6.7	9.9×9.9
Virion membrane glycoprotein	48K	7.8	12.8×8.8	50K	6.2	10.2×3.8	53K	6.8	10.4×9.9
					6.3	10.8×3.8			
E2 50K	49K	7.6	12.4×8.5	46K	≥8.0	15.2×4.2		ND	
Virion membrane glycoprotein	49K	7.8	12.8×8.5						

ND=Not detected

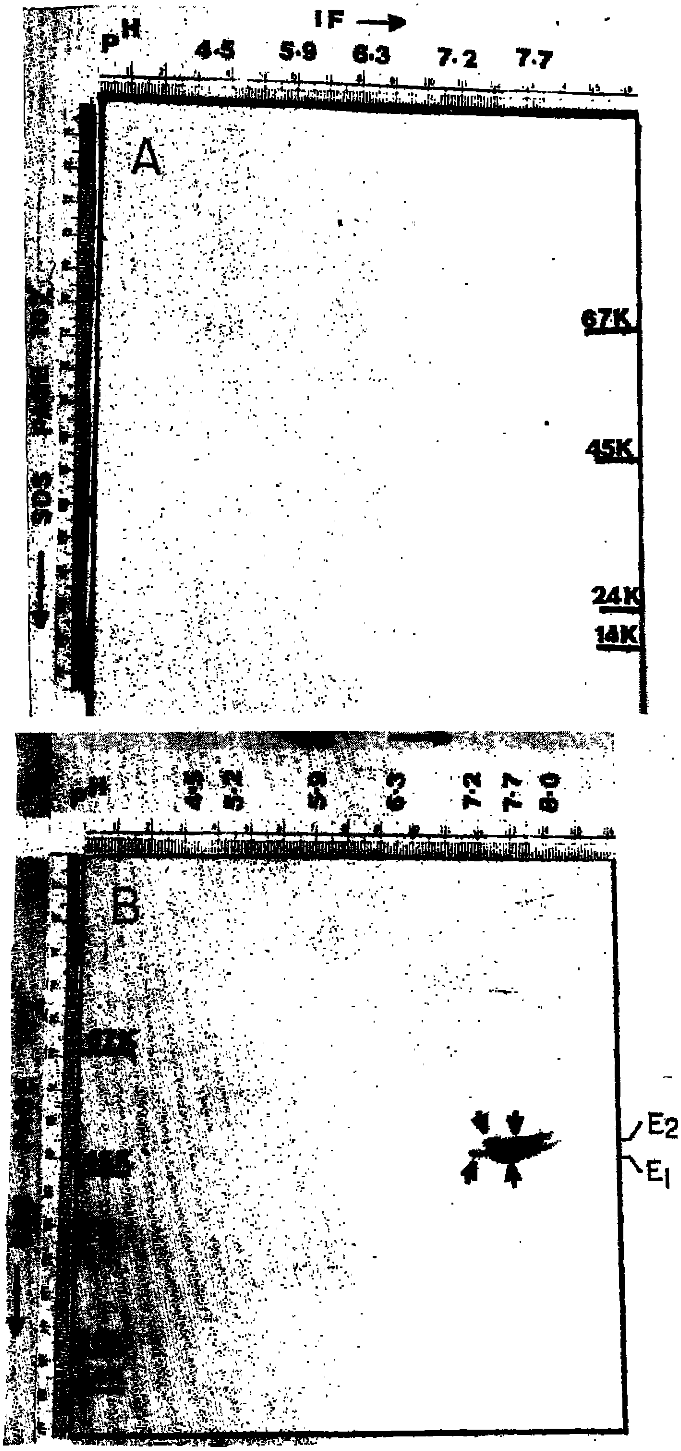

Figure 2. (*Captions in p. 198*).

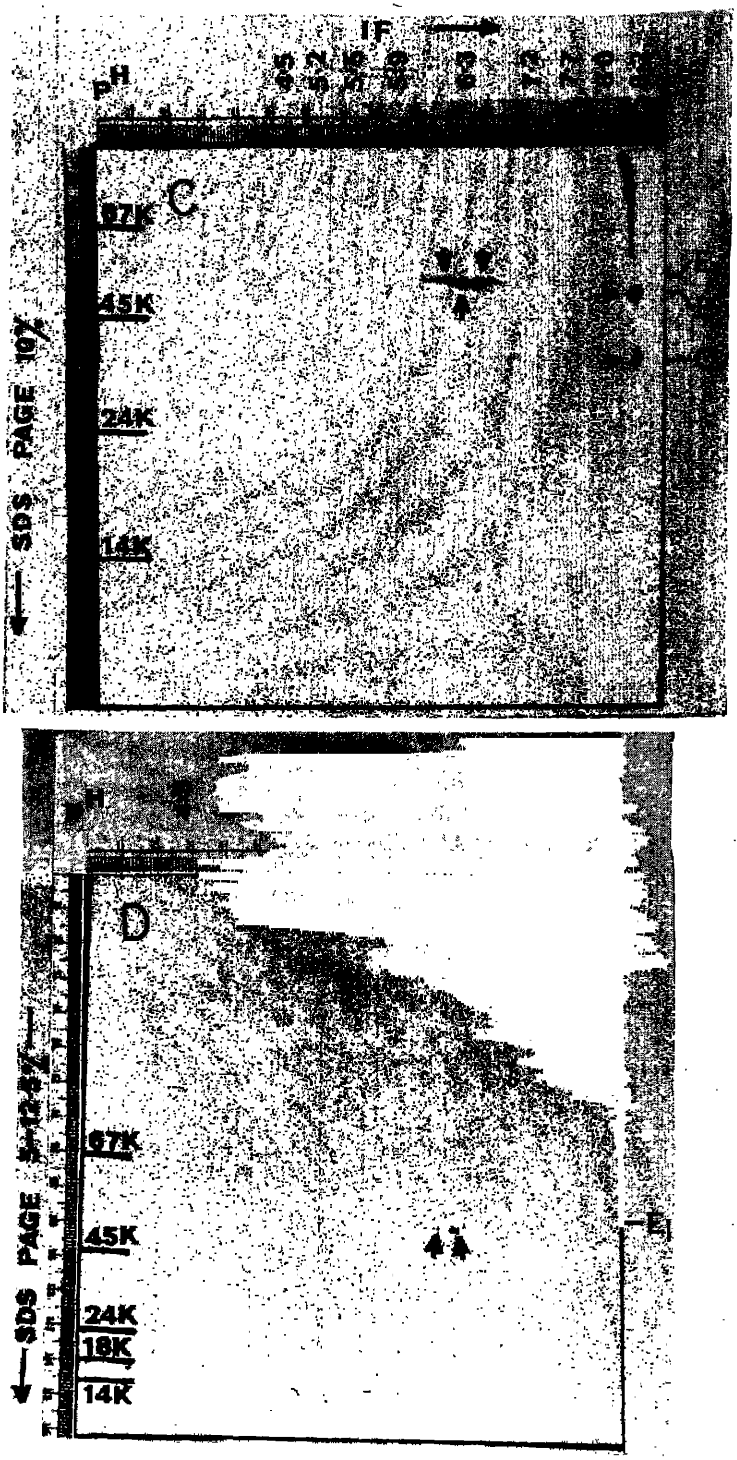

Figure 2. Two dimensional polyacrylamide gel electrophoresis of purified alphaviruses. Alphaviruses were grown on Vero cells and radiolabeled with $[^3H]$-amino acids. Virus purification procedure, and electrophoretic conditions were as described in the text. **A.** Purified mock. **B.** Purified SF virus. **C.** Purified SIN virus. **D.** Purified CHIK

of SIN virus. Therefore, the acidic nature of capsid protein (pI 4) reported by Dalrymple *et al.* (1976) seems to be unlikely.

The E1 and E2 of SIN and Western equine encephalitis (WEE) virus are of pI 6.0 and pI 9.0 respectively (Dalrymple *et al.*, 1976; Hashimoto and Simizu, 1979). SF virus is closely related to SIN and WEE viruses, but interestingly enough the E1 and E2 of SF virus have almost identical isoelectric points. Each of E1 and E2 of SF virus had two subspecies with pI 7.4, 7.8 and pI 7.6, 7.8 respectively (table 2).

It has been reported (Dalrymple *et al.*, 1976; Hashimoto and Simizu, 1979) that isoelectric-focusing can be successfully applied to separate envelope glycoproteins E1 and E2 of detergent disrupted virions of SIN and WEE. The separation being possible of the marked differences in the isoelectric points of these proteins. However, our observation that E1 and E2 of SF virus share common isoelectric points, explains why earlier effort to separate these glycoproteins of SF virus by isoelectric focusing (Garoff *et al.*, 1974) were unsuccessful.

Strong non-covalent interaction between E1 and E2 of alphaviruses has been proposed (Rice and Strauss, 1982) to explain how host proteins are excluded from the virion during budding process (Strauss, 1978) and how equimolar stoichiometry of structural proteins in the mature virions is achieved (Schlesinger *et al.*, 1972; Rice *et al.*, 1982). From our study it appears that the interaction between E1 and E2 may be independent of the charges they carry, for E1 and E2 have almost identical charges in case of SF virus, while in case of SIN virus, E1 and E2 have widely different charges.

Multiple subspecies of P62 polypeptide of SF virus observed in our study may represent different steps of post-translational modifications (glycosylation, phosphorylation and sulphation) through which P62 has to pass before achieving its final mature form. During the process of glycosylation it has been observed that initial glycosylation takes place at rough endoplasmic reticulum (Wirth *et al.*, 1977) leading to mannose rich glycosylation (Robbins *et al.*, 1977; Schwarz 1978), which is followed by removal of excess amounts of mannose by a process called 'trimming' (Klenk and Rott, 1980). Gradual decrease in molecular weights observed in P62 of SF virus could be due to the residual intermediate 'trimmed' forms of P62; or otherwise it could be an indication of stepwise addition of peripheral sugars (Sefton, 1977) leading to slight increase in molecular weight accompanied by sight change in its isoelectric point at each addition. However, we have found that the mature form of P62 which we believe, represented by the darkest spot on fluorograph, was of the lowest molecular weight of all forms of P62. This observation seems to indicate that multiple spots of P62 with gradual decrease in their molecular weights may represent the removal of mannose from intially glycosylated product of P62 during the 'trimming' process.

The vertical streak of P62 (figure 1B) with SF virus infected cells represents species of P62 with different molecular weights but having identical isoelectric point. The heterogeneity in molecular weight of P62 has also been reported earlier (Kaluza, 1975). The heterogeneity may be due to different amounts of neuraminic acid on the complex chains (Burke and Keegstra, 1976), or to the differences in the core structures (Klenk and Rott, 1980).

A polypeptide 57 K (pI 7.8) which could be distinctly resolved from P62 in 2 D PAGE of SF virus infected cell lysates may represent nonstructural protein ns60, which otherwise, comigrated in the conventional Laemmli's gels (Kaariainen and Soderlund, 1978).

It was of interest to find that E1 of CHIK virus in infected cells resolved into three isoproteins (pI 6.3, 6.5 and 6.7), but E1, from purified virus preparation contained only two. This suggests that during assembly and maturation process, there is a preferential selection of two out of the three isoproteins of E1, synthesised in infected cells.

Charge heterogeneity in the membrane glycoproteins could be due to post translational modifications. At present, it is not possible to state which type of post translational event is responsible for the charge heterogeneity. It is even not clear, whether such a charge heterogeneity is an accidental event, resulting from non-specific action of cellular enzymes, involved in the process of post translational modifications, or is a deliberate event, resulting from some unknown virus specific control.

There may be a correlation between charge heterogeneity and the complexity of the virus. Thus while flaviviruses, which contain a single major membrane glycoprotein, fail to exhibit charge heterogeneity (Deshmane and Banerjee, unpublished observation), and alphaviruses, which contain 2-3 membrane glycoproteins, show limited charge heterogeneity (present report), the relatively complex rhabdoviruses, orthomyxoviruses and paramyxoviruses show more charge heterogeneity in their glycoproteins (Rhaghow *et al.*, 1978; Privalsky and Penhot, 1978; Leavitt *et al.*, 1979).

In these experiments the individual proteins were located by careful comparison with the mock infected controls. This however has a subjective bias. An ideal solution would be to employ monoclonal antibodies for specific identification.

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Colchicine binding activity of rat brain polysomes

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Abstract. In this communication, we report the presence of a unique colchicine-binding activity in the polysomes of rat brain. This drug-binding property, is somewhat similar to that of tubulin isolated from many sources; however, it differs in several biochemical characteristics such as (i) thermal stability of colchicine-binding site, (ii) protection of binding site by vinblastine and (iii) time required for binding equilibration. Such binding of colchicine to the polysomes is most probably due to the presence of a nascent peptide chain of tubulin in the polysome.

Keywords. Colchicine-binding activity; tubulin polysomes; nascent peptide chain.

Introduction

Colchicine binds to tubulin, the subunit of microtubule with a stoichiometry approaching one mol of alkaloid per mol of tubulin dimer. This unique binding specificity of colchicine.tubulin interaction which is comparable to enzyme-substrate reaction has been routinely employed to characterise tubulin (Olmsted and Borisy, 1973; Wilson and Bryan, 1974; Bhattacharyya and Wolff, 1974, 1975, 1976, 1976a). The ubiquitous and multifunctional nature of tubulin commends it for studies on the regulation of its biosynthesis and function. Vertebrate brain constitutes a rich source of tubulin and is very likely to contain also an appreciable amount of tubulin-specific messenger RNA. The approach for the isolation of tubulin mRNA is still very limited except for a few reports (Cleaveland *et al.*, 1978; Gozes *et al.*, 1980; Portier *et al.*, 1980). The purification of a specific mRNA requires selection of specific polysomes with which the mRNA of the desired proteins is associated. In the present study, colchicine is used to detect the tubulin synthesizing polysomes isolated from rat brain. This colchicine binding property of the brain polysomes is characterized in order to compare with that of tubulin itself, the protein isolated from many sources.

Materials and methods

Radioactive colchicine (ring C[^{3}H]methoxy) having specific activity 5 Ci/mmol was the product of New England Nuclear Corpn., Boston, Massachhusetts, USA.

Abbreviations used : TNMG, Tris-HCl buffer pH 7.2, 25 mM NaCl, 5 mM $MgCl_2$ and 0.1 mM GTP; UV, ultra violet; DEAE, diethyl aminoethyl.

GTP (Grade IIS) and colchicine were products of Sigma Chemical Co., St. Louis, Missouri, USA. Colcemid was a kind gift from Dr. Jan Wolff of National Institutes of Health, Bethesda, Maryland, USA. DE-81 and GF/C filter papers were obtained from Whatman Inc., UK. All other chemicals used were reagent grade.

Polysomes were isolated from rat brain (1 month old) according to the method of Pemberton *et al.* (1972) with little modifications. Excised brain was homogenized (4 ml/g) with 10 mM Tris-HCl buffer (pH 7.4), 10 mM KCl and 1 mM $MgCl_2$. The crude homogenate was mixed with 1/3 volume of 2.4 M sucrose, 170 mM Tris-HCl buffer (pH 7.4), 70 mM KCl and 1 mM $MgCl_2$. The nuclei were removed by centrifugation at 5000 *g* or 10 min. The postnuclear supernatant was centrifuged at 30,000 *g* for 10 min to remove mitochondria. Free polysomes were recovered by centrifugation of the postmitochondrial supernatant at 100,000 *g* for 3.5 h through a sucrose cushion contaning 1.6 M sucorse, 50 mM Tris-HCl buffer (pH 7.4), 250 mM KCl and 2 mM $MgCl_2$.

Binding of [^3H]colchicine to rat brain polysomes

The binding of labeled colchicine to polysomes was quantitated by the method of Bhattacharya and Wolff (1975). DEAE-filter paper discs were initially washed with 1 ml of cold (2-4°C) PM buffer (10 mM potassium phosphate, pH 7.0, and 10 mM $MgCl_2$), taking care not to dry the papers. Then, 1 ml of the buffer containing 10 μm colchicine was poured on the filter paper to which 100 μl of the sample was added and allowed to drain off under mild suction. The filters were then rinsed 3 times with 3 ml of the cold buffer by mild suction, dried, and counted in 5 ml of toluene-based fluor. Identical blanks, without polysome, were prepared and necessary corrections were made.

Results

Polysome profile

The polysomes were suspended in TNMG buffer (25 mM Tris-HCl buffer, pH 7.4, 25 mM NaCl, 5 mM $MgCl_2$ and 0.1 mM GTP), layered over a linear sucrose (15–30%) gradient and centrifuged at 25,000 *g* for 90 min. Fractions were collected using an ISCO Model 183 fractionator and the absorbance at 254 nm was recorded with an UA-5 ultra violet (UV) monitor. The profile of the polysomes shows the integrity of the isolated polysomes (figure 1). Preliminary binding experiments with this purified preparation suggest that like tubulin from several sources polysomes isolated from brain, bind [^3H] colchicine (table 1) and that the binding activity is a linear function of the polysome concentration (figure 2a) with a temperature optimum at 37° C (figure 2b). The specificity of this binding is also similar to that of tubulin from brain and other sources (Olmsted and Borisy, 1973; Wilson and Bryan, 1974; Wolff and Williams, 1973). Thus, the binding of colchicine to brain polysomes can be completely blocked, if the polysomes are preincubated with other drugs, that are known to bind specifically at the colchicine-binding site of tubulin (table 1). However, unlike colchicine-tubulin interaction which is a slow process and requires 2 h to attain equilibrium at 37° C, the rat brain polysomes bind colchicine more rapidly and equilibrium is attained within 45 min (data not shown). Although, the soluble tubulin constitutes the bulk of the colchicine-binding activity of many tissues (Borisy and Taylor, 1967a, b), it has

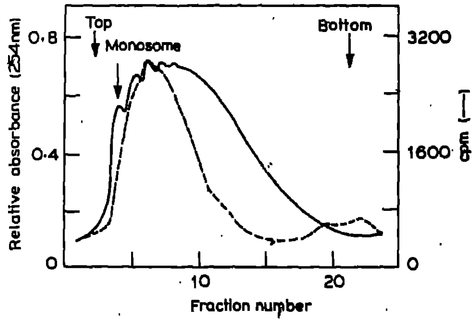

Figure 1. Sedimentation profile of the polysomes. Approximately 70 A_{280} units in 0.5 ml were applied on 15–30% sucrose gradients. Centrifugation was at 25,000 rpm for 90 min at 0° C in a Spinco ultracentrifuge.

Table 1. Effect of colchicine analogues on [^{3}H]colchicine binding to rat brain polysomes.

Experiments	[^{3}H] colchicine binding activity (cpm/2.08 A_{280} units)
Control	1329
Preincubated with colchicine (0.1 mM)	93
Preincubated with colcemid (0.1 mM)	128
Preincubated with podophyllotoxin (8.12 mM)	83

Polysomes (5.2 A_{280} units) were preincubated with drugs for 10 min at 37° C and the colchicine binding activity were measured by further incubating the samples with [^{3}H]colchicine (1 μM) at 37° C for 30 min.

been shown that the colchicine-binding protein may also be associated with the particulate cell fractions (Feit and Barondes, 1970; Dahl *et al.*, 1970; Lagnado *et al.*, 1971; Stadler and Franke, 1972). Thus, the question arises whether this colchicine-binding activity, residing in the brain polysomes, is due to a specific association with nascent tubulin, or due to some nonspecific adsorption of cytoplasmic soluble tubulin or plasma membrane bound tubulin during cell homogenization. To test these possibilities, cytoplasmic tubulin-[^{3}H] colchicine complex was added before homogenizing the brain and polysomes were prepared in the usual procedure. There was no detectable radioactivity in the polysomes after separation through a discontinuous sucrose gradient (data not shown). This result eliminates the possibility of contamination of the polysomes with the cytoplasmic tubulin. Furthermore, since the concentration of sucrose cushion (1.6 M) used in the isolation of polysomes is more than 45%, it seems unlikely that the colchicine-binding activity of the polysomes was due to the contamination by the plasma membrane or an artifact of separation (Bhattacharyya and Wolff, 1975).

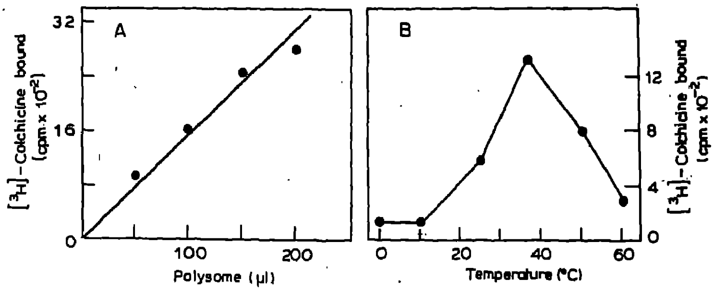

Figure 2. Binding of [^{3}H]colchicine to rat brain polysomes. **A.** Linearity of the [^{3}H] colchicine-polysome binding reaction. [^{3}H]Colchicine (1 μm) was incubated with increasing concentrations of polysomes in a final volume of 250 μl, at 37° C for 30 min and the binding was monitored as described in methods. **B.** Temperature dependence of [^{3}H] colchicine-polysome binding reaction. Aliquots of polysome (5.2 A_{260} units) in a final volume of 250 μl were incubated with [^{3}H] colchicine (1 μm) at different temperatures for 30 min and colchicine binding was measured.

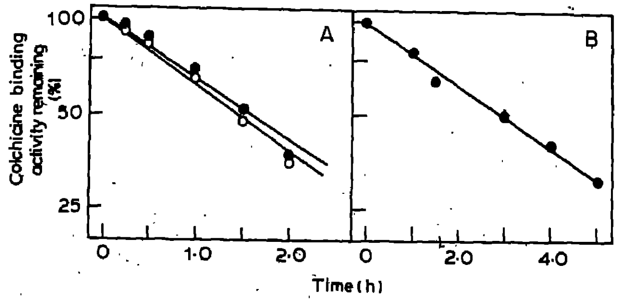

Figure 3. Time decay of colchicine-binding activity of rat brain polysomes. **A.** Aliquots of polysomes (5.2 A_{260} units) in a final volume of 250 μl were incubated in the absence (○) or in the presence (●) of 10 μM vinblastine sulphate at 37° C for various periods. Colchicine binding activity of the preincubated samples were determined by further incubating with [^{3}H] colchicine 1 μM at 37° C for 30 min. Data were expressed as the per cent binding activity remaining compared to the sample without preincubation as 100%. **B.** Aliquots of polysomes (5.2 A_{260} units) were incubated at 0° C for various periods. Colchicine binding activity of the samples were determined by further incubating the samples with [^{3}H]colchicine 1 μM at 37° C for 30 min.

An interesting property of the colchicine-binding site of tubulin is its lability at 37° C. Figure 3a shows the decay characteristics of colchicine-binding activity of polysomes at 37° C. In comparison to soluble and membrane-bound tubulins (Bhattacharyya and Wolff, 1975), the colchicine-binding activity of the brain polysomes is more liable at 37° C. The time required for the loss of 50% binding activity over the control has been found to be only 90 min whereas the values for the soluble and the membrane bound tubulins are 4 h and 7 h respectively (Bhattacharyya and Wolff, 1975). Moreover, vinblastine which is known to protect the colchicine-binding site of tubulin (Bhattacharyya and Wolff, 1975; Wilson, 1970), fails to stabilise the colchicine-binding activity of the polysomes (curve 2). It has already been known that tubulin isolated from different sources is quite stable at 0° C with respect to the colchicine binding. But surprisingly this polysomal colchicine-binding activity is so labile that it decays even at 0° C (figure 3b), showing a half-life of 3 h. It seems, therefore that the colchicine-binding activity of polysomes is not due to any contamination of soluble or membrane bound tubulin. Such binding of colchicine to the polysomes is most probably due to the presence of nascent peptide chain of tubulin in the polysome. However, our efforts to release the nascent chain by puromycin treatment to show colchicine binding activity failed. Since the colchicine binding activity of polysome is highly labile compared to cytoplasmic tubulin (figure 3b), it is possible that puromycin released subunit quickly lost its conformation (needed for the colchicine binding activity) when present in a monomeric form.

Discussion

In this communication, we report the presence of a unique colchicine-binding activity in the polysomes of rat brain. This drug-binding property, is somewhat similar to that of tubulin isolated from many sources; however, it differs in several biochemical characteristics such as (i) thermal stability of colchicine-binding site, (ii) protection of binding site by vinblastine and (iii) time required for binding equilibration. Such binding of colchicine to the polysomes is most probably due to presence of nascent peptide chain of tubulin in the polysome. However, it is not possible to suggest as to whether α or β subunit binds colchicine *per se*. After addition of colchicine before fractionation of polysomes it is noted that the profile of colchicine binding remained the same (data not given). Had it been due to both the subunits, a shifting of colchicine binding profile of polysomes towards bottom should have been noted. Nevertheless, the most important finding which emerges from this study is that since the polysome-bound nascent peptide binds colchicine and α and β subunits of tubulin are coded by two different genes as reported by Valenzuela *et al.* (1981), the colchicine-binding site of tubulin is expected to be located in one of the subunits. The present results support this fact. Recently similar conclusion was also drawn by Rousset and Wolff (1980) on the basis of their studies on lactoperoxidase-tubulin interaction. Finally, colchicine-binding of polysome might be used as a probe for isolation of specific tubulin mRNA.

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Effect of denaturants on the 11S protein of guar seed (*Cyamopsis tetragonoloba*)

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Abstract. The effect of sodium dodecyl sulphate, urea or guanidinium hydrochloride on the sedimentation velocity, viscosity, ultra violet spectra and fluorescence spectra of the 11S protein of guar seed has been determined. Sodium dodecyl sulphate dissociates the protein directly to the 2S protein, whereas urea or guanidinium hydrochloride produces an intermediate 7S protein. These reagents denature the protein also. Both the dissociative and the denaturation effect follow the order, sodium dodecyl sulphate > guanidinium hydrochloride > urea when the concentration are expressed as mols per litre. The denatured states in the three cases probably differ.

Keywords. Denaturation; guar seed; 11S protein.

Introduction

Guar bean (*Cyamopsis tetragonoloba*) is a commercially important crop of India and serves as the raw material for the industrial production of the galactomannan gum (guar gum). Guar meal obtained after the extraction of the gum contains about 45% protein and can be a valuable proteinaceous raw material (Subramanian and Parpia, 1975).

We have been studying the proteins of guar seed with the object of characterising them. In sedimentation velocity experiment, the 1 M NaCl extract of guar meal, after dialysis, gives three peaks with $S_{20,w}$ values of 1.5, 6.4 and 9.5 S; the last fraction forms 30% of the total (Nath *et al*, 1978). A method has been developed to isolate the 9.5 S protein in a homogeneous form (Nath *et al*,1980). Whereas with the total protein $S_{20,w}$ obtained for the protein was 9.5 S, the isolated fraction gave a value of 10.5 S, at 1% protein concentration (Nath *et al*, 1980). We designate it as the 11S protein of guar seed.

The molecular weight (M_r) of the 11S protein determined by $S_{20,w}$ and intrinsic viscosity was 228,000 daltons. In sodium dodecyl sulphate—polyacrylamide gel electrophoresis (SDS—PAGE) it gave at least six non-identical polypeptide chains (Nath *et al*, 1980). Since the 11S protein was oligomeric, the effect of denaturants on the protein has been determined.

Abbreviations used: SDS, Sodium dodecyl sulphate; GdmCl, guanidinium hydrochloride; PAGE, polyacrylamide gel electrophoresis; DEAE, diethyl amino ethyl; M_r, molecular weight; RTIC, rotor temperature indicator and control.

Materials and methods

Materials

Guar seed: Guar seed of the variety FS-277 was obtained from the Haryana Agricultural University, Hissar.

Chemicals: Guanidinium hydrochloride (GdmCl) was from Sigma Chemical Co., St. Louis, Missouri, USA, urea from British Drug House, and SDS from Hindustan Lever Co., Bombay. Urea was recrystallised once from 50% ethanol. All the other chemicals were of analytical reagent quality.

Methods

Isolation of the 11S protein: The procedure described previously was used (Nath *et al*., 1980).

Protein concentration: This was determined by measuring the absorbance at 280 nm and using the experimentally-determined value of 7.0 for $E_{1}^{1\%}$ cm.

Ultracentrifugation: The experiments were performed at room temperature (~28° C) using 1% protein solution in the appropriate solvent. Centrifugation was carried out at 6.6×10^5 *g* in a Spinco Model E analytical ultracentrifuge equipped with rotor temperature indicator and control (RTIC) unit and phase-plate schlieren optics. From the photographs taken at different intervals of centrifugation, the apparent sedminatation coefficient (S_{app}) was calculated. From enlarged tracings, the proportion of various protein components was calculated.

Viscosity: The measurements were made at 30±0.1°C in an Ostwald Viscometer having a flow time of 180 s with distilled water. A 1% protein in the appropriate solvent was used.

Difference spectra: Difference spectra produced by the addition of different concentrations of the denaturant were recorded in a Perkin Elmer double-beam recording spectrophotometer model 124, in the range 240 to 300 nm. Protein solutions having an absorbance of 1.6 at 280 nm were used. Since urea and GdmCl solutions had absorbance in the region below 300 nm, the absorbance value was subtracted from ΔA value at that concentration.

Fluorescence: The emission spectrum of the protein solution having an absorbance of 0.07 at 280 nm was measured at room temperature in the range 300–400 nm after excitation at 280 nm. The fluorescence of the solvent blank was measured and subtracted from that of the protein. A Perkin Elmer fluorescence spectrophotometer model 203 was used.

Other measurements: The pH measurements were made with a Radio-meter pH meter TTT 2. Absorbance measurements were made with a Carl-Zeiss ultra violet spectrophotometer.

Results and discussions

Unless otherwise stated, all the measurements were made in 0.02 M phosphate buffer solution of pH 7.5. The effect of denaturants monitored by different techniques was time-dependent upto 3 h, after which no change in the measured values occurred. Therefore, the protein prepared in the denaturant solution was kept at room for 3 h and used subsequently for the measurement. The concentration of the denaturant mentioned is total concentration and not free concentration.

The 11S protein was homogeneous by the techniques of gel filtration and diethyl amino ethyl (DEAE) cellulose chromatography. However, on gel electrophoresis and ultracentrifugation, it showed the presence of a small proportion (<5%) of a high M_r protein. The sedimentation velocity pattern obtained in 1 M NaCl or 0.02 M phosphate buffer solution was the same suggesting that salt had no associative or dissociative effect on the protein.

Upto a concentration of 3.5 mM, SDS had no effect on the sedimentation velocity pattern of the 11S protein. Above this concentration 2 peaks of S_{app} values of 11 and 2S appeared (figure 1B). With increase in SDS concentration the proportion of the latter increased while that of the former decreased (figure 1C and D) and at 17.3 mM SDS, 2S peak alone was observed (figure 1E).

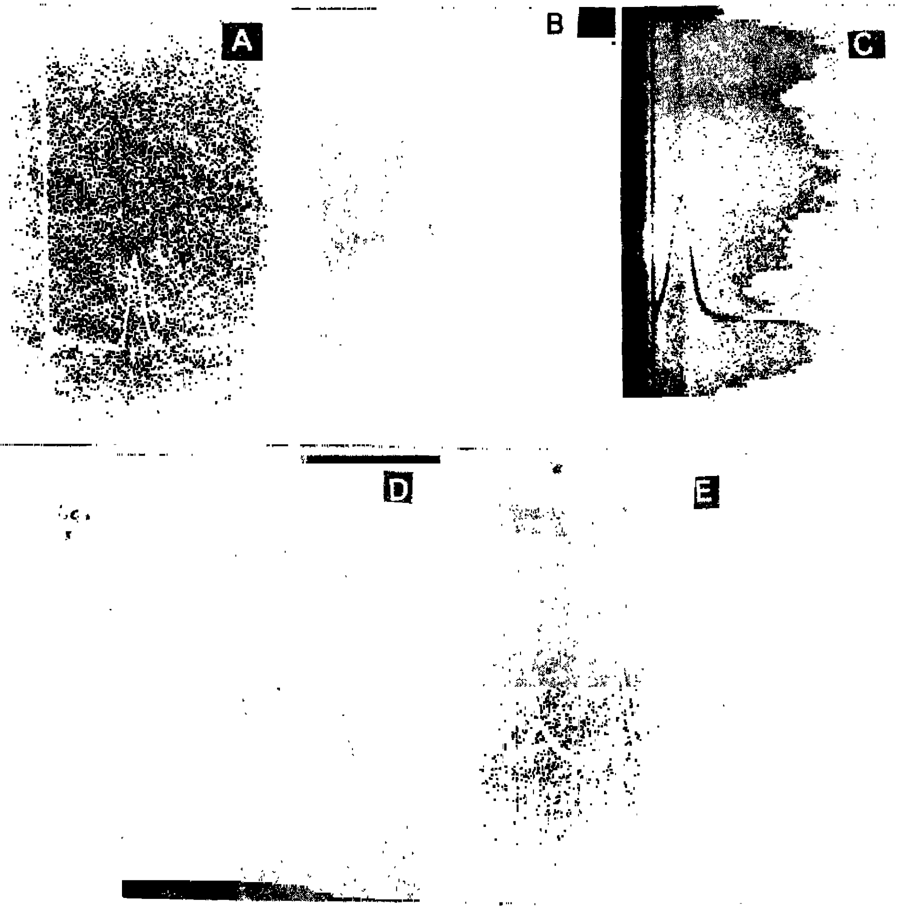

Figure 1. Sedimentation velocity pattern of guar 11S protein in presence of SDS. **A.** 0 SDS; **B.** 3.5 mM SDS; **C.** 5.2 mM SDS; **D.** 8.7 mM SDS; and **E.** 17.4 mM SDS.

In urea and GdmCl solution the following was observed in sedimentation velocity experiments. Urea at 1 M concentration had no dissociative effect. At 2 M and 3 M urea the solution developed turbidity. When these solutions were centrifuged in the analytical ultracentrifuge, a peak appeared around 9,800 *g* which sedimented rapidly and the solution became clear. At higher concentrations turbidity and peak formation at 9,800 *g* were not observed. Three peaks with S_{app} of 11S, 7S and 2S were observed (figure 2A, B and C). At 7 M urea concentration, .2S peak alone was observed (figure 2D).

Dissociation of the 11S protein into 7S and 2S was observed in GdmCl solutions also (figure 3). With this denaturant, turbidity did not develop at any concentration. Also dissociation occurred at lower concentrations than with urea.

In their dissociative effect on the 11S protein, SDS and urea (or GdmCl) showed a marked difference. SDS dissociated the 11S protein directly to 2S protein whereas urea or GdmCl dissociated through an intermediate 7S protein. Also with SDS, dissociation into 2S protein was complete at 17.3 mM SDS concentration. SDS is tightly bound to proteins, possibly by ionic and hydrophobic bonds (Steinhardt and Reynolds, 1969). If such forces are responsible for the oligomeric

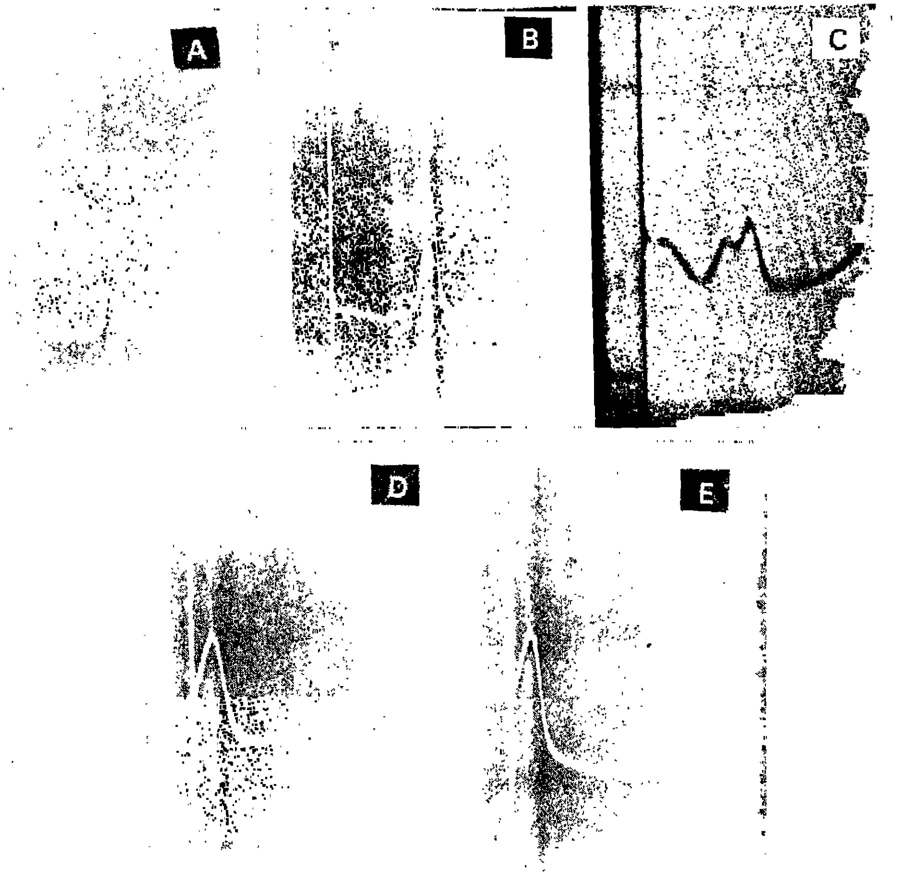

Figure 2. Sedimentation velocity pattern of guar 11S protein in presence of urea. **A.** 0 urea; **B.** 1 M urea; **C.** 2 M urea; **D.** 5 M urea and **E.** 7 M urea.

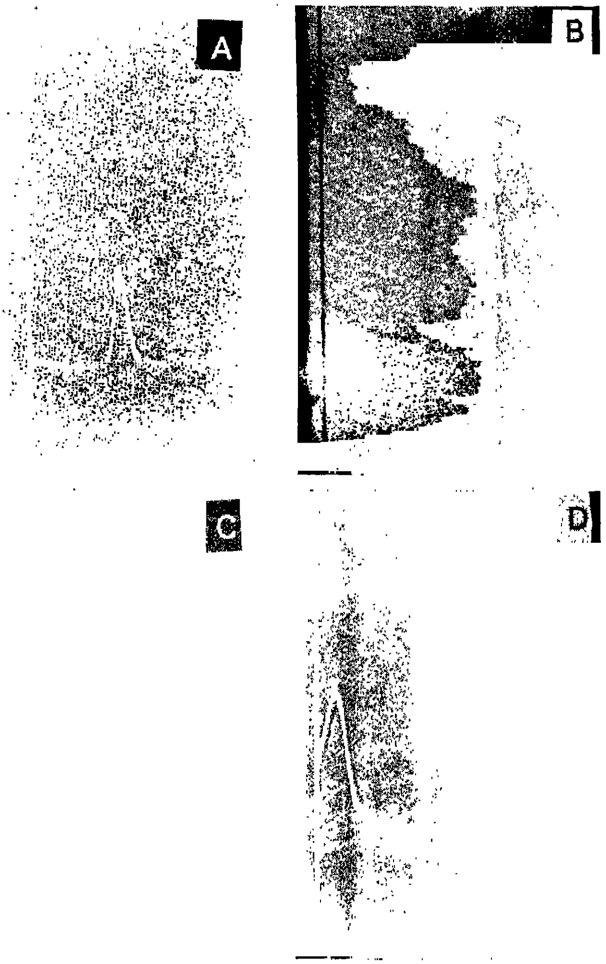

Figure 3. Sedimentation velocity pattern of guar 11S protein in presence of GdmCl. **A.** 0 GdmCl; **B.** 1 M GdmCl; **C.** 2 M GdmCl; and **D.** 3.5 M GdmCl.

structure of the 11S protein, dissociation can be achieved by breaking them. Urea or GdmCl can break only φ and amide/peptide bond (Nozaki and Tanford, 1963). Because of its ability to break ionic and hydrophobic bonds SDS may be expected to exert greater dissociative effect than urea or GdmCl. Also the conformational stability of the 7S protein may be poor, and it may dissociate into 2S protein as soon as it is formed.

There were three observable differences in the effect of urea and GdmCl on the 11S protein: (1) in 1 M urea solution the 11S protein developed turbidity and formed an aggregate which sedimented at ~9,800*g*; no such effect was observed in GdmCl solution, (2) the dissociative effect of GdmCl was greater than that of urea. At 3.5 M GdmCl the 11S protein had completely dissociated to the 2S protein,whereas at the same concentration of urea, the proportion of the 2S protein was only 70% (figure 4) and (3) in urea solutions the proportion of the 7S peak

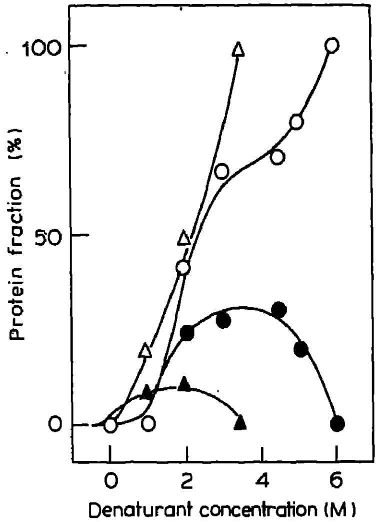

Figure 4. The proportion of 7S and 2S proteins as a function of denaturant concentration. 2S in urea, (○); 7S in urea, (●); 2S in GdmCl, (△); 7S in GdmCl, (▲).

reached a maximum value of ~30% and in GdmCl solution it reached only a value of ~10%. These suggest the greater dissociative effect of GdmCl, an observation which agrees with those on other proteins (Green and Pace, 1974).

In its behaviour in SDS, urea or GdmCl solution, the guar 11S protein resembles the 11S protein of sunflower seed (*Helianthus annuus*). SDS dissociates the 11S protein of sunflower to 2S protein directly, whereas urea or GdmCl dissociates it to 7S and 2S protein, the ultimate dissociated from, being the 2S protein (Rahma and Narasinga Rao, 1981).

In 1 M urea solution a fast-sedimenting fraction, possibly an aggregate, was observed. Prakash and Nandi (1977) have reported the formation of a fast-sedimenting protein aggregate of sesame α-globulin in urea solution of low molarity.

The effect of the denaturants was also monitored by viscometry, ultra violet (UV) difference spectra and fluorescence spectra. The η_{red} of the protein in 0.01 M sodium phosphate buffer of pH 7.5 was 3.8 cc/g at 1% protein concentration. Addition of SDS increased the viscosity and a maximum value of 8.4 cc/g was observed at and above 9 mM SDS concentration (figure 5 inset). Urea and GdmCl also increased the value of η_{red} (figure 5). The maximum value obtained in urea solution was 22 cc/g and in GdmCl 29 cc/g. In SDS solution the value was only 8.4 cc/g. It has been shown that in 6 M GdmCl solution, most proteins exist in fully random-coil conformation (Lapanje, 1978). The maximum η_{red} values obtained in 7 M urea and 17 mM SDS solutions were lower than that in 6 M GdmCl solution suggesting that in the first two solvents complete random-coil conformation was not attained.

The addition of the denaturant to the protein solution caused a difference spectrum to appear in 250–300 nm region. Typical spectra produced by the addition of SDS are given in figure 6. Troughs at 292 nm, 287 nm and 278 nm were observed with increase in SDS concentration; the intensity of the troughs increased with SDS concentration. Similar difference spectra were produced by urea or GdmCl.

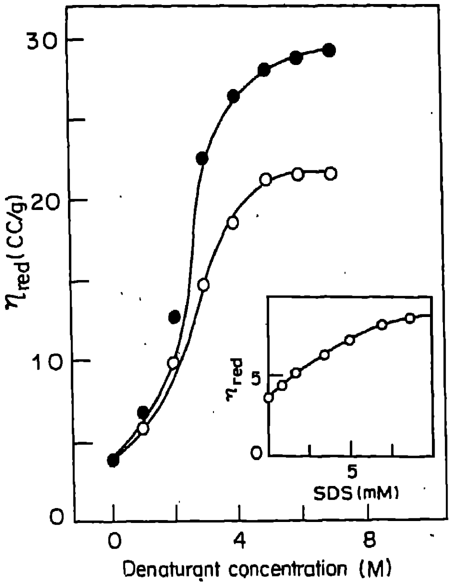

Figure 5. Reduced viscosity of guar 11S protein in presence of denaturants. Urea, (O); GdmCl, (●). Inset: SDS.

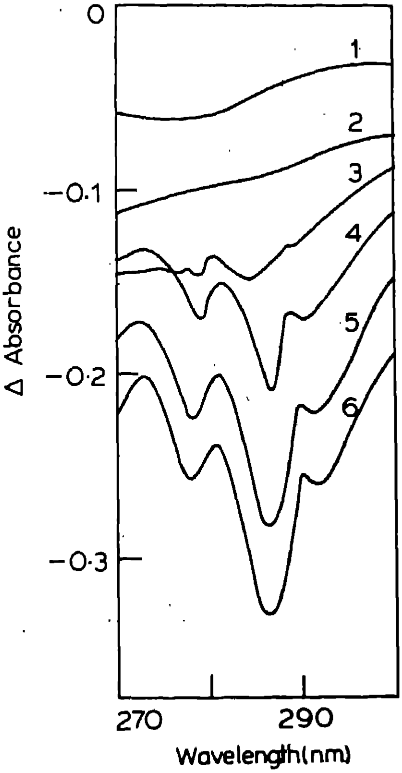

Figure 6. Difference spectra produced by the addition of SDS to guar 11S protein. (1) 0.35 mM; (2) 0.87 mM; (3) 1.73 mM; (4) 3.5 mM; (5) 8.7 mM; (6) 17.4 mM SDS.

The blue shift produced by the denaturants is due to the perturbation of tyrosine and tryptophan residues (Donovan, 1969) and the effect of the 3 denaturants appeared to be similar.

The addition of the denaturants quenched the fluorescence intensity of the protein and also caused a red shift of the emission maximum. Typical spectra produced by the addition of GdmCl solution are given in figure 7. The protein has emission maximum at 325 nm which is characteristic of tryptophan emission (Teale, 1960). Fluorescence quenching increased with GdmCl concentration and there was a red shift in emission maximum. These are characteristic of protein denaturation (Chen *et al*., 1969). Similar effect was produced by SDS and urea also.

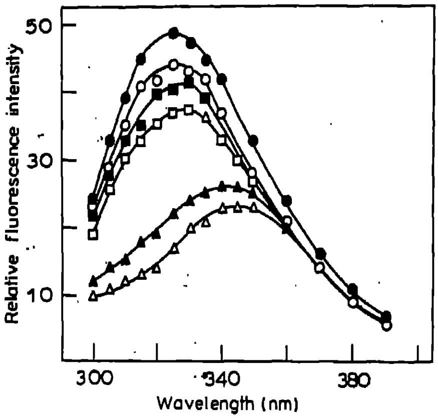

Figure 7. Fluorescence emission spectrum of guar 11S protein in presence of GdmCl. No GdmCl, (●); GdmCl 1 M, (○); GdmCl 1.5 M (■); GdmCl 2 M, (□); GdmCl 3M, (▲); and GdmCl 4 M, (△).

The results of sedimentation velocity measurements indicated the dissociation of the protein and those of viscosity and spectral measurements denaturation of the protein, by SDS, urea or GdmCl. The effect of SDS appeared to be greater than that of urea or GdmCl, since dissociation and denaturation occurred at much lower concentrations of SDS.

Using the viscosity, difference spectral and fluorescence spectral data, the fraction of protein denatured as a function of denaturant concentration was calculated by the standard procedure (Tanford, 1968). Such data for GdmCl are given in figure 8. Similar profiles were obtained in the case of SDS and urea.

From figure 8A it may be seen that the profile consisted of a single step and the curve was steep. Also the values obtained by different techniques fit the same curve. These are taken as evidence for the interpretation that the denaturation reaction is a single step process without the presence of any intermediates and is cooperative (Pace, 1975).

Further it was also observed that the data of urea denaturation fitted the curve in figure 8A, thus suggesting that the extent of denaturation was the same in both the solvents. However, reference to figure 5 shows that the maximum value of η_{red} in urea is ~22 cc/g and that in GdmCl ~29 cc/g. Also in difference spectra the ΔA values were higher in the case of GdmCl than in urea; similarly the quenching of fluorescence was higher in GdmCl. These results suggest that the protein is more fully denatured in 6 M GdmCl solution than in 6 M urea or 17 mM SDS solution.

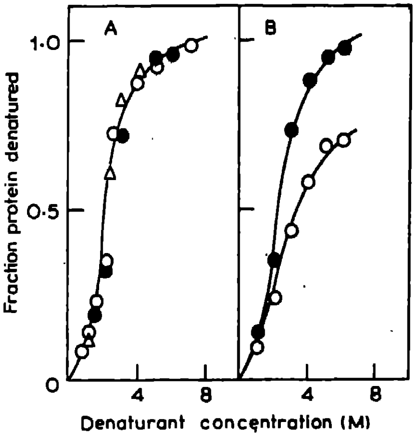

Figure 8. **A.** Fraction of guar 11S protein denatured as a function of denaturant concentration (data in GdmCl). Viscosity data, (O); fluorescence data, (Δ); and difference spectral data, (●). **B.** Fraction of guar 11S protein denatured as a function of denaturant concentration (from viscosity data). Urea, (O); and GdmCl, (●).

The fraction of protein denatured is calculated from the limiting values of the viscosity, difference in absorbance and extent of quenching at the highest concentration of the particular denaturant; these limiting values differ in GdmCl and urea solution. But the fraction calculated may fit fortituously the same curve in both the cases.

The maximum values found in 6 M GdmCl solution mav be taken to correspond to those of the fully denatured protein (Lapanje, 1978). If these values are used to calculate the fraction of protein denatured in urea solutions, the data do not fit the curve of GdmCl data (figure 8B). The extent of denaturation was less in urea solutions.

It should be possible to determine if the denatured state of the 11S protein in 6 M GdmCl solution is the same, or not, by optical rotatory dispersion and circular dichroism methods. Such work is in progress.

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Proteases in germinating finger millet (*Eleusine coracana*) seeds

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Abstract. Proteolytic activity was estimated in germinated finger millet seedlings using the endogenous trypsin/amylase inhibitor as substrate and also with haemoglobin and albumin as substrates. The maximal proteolytic activity was observed on the third day of germination. With the inhibitor as substrate, the proteolytic activity was maximal at pH 2.5. The protease that acted on the inhibitor required sulphydryl groups for maximal activity and was suppressed by diazoacetyl norleucine methyl ester and Pepstatin. The protease that acted on haemoglobin with optimum pH of 5.0, was more stable on storage, did not depend on sulphydryl groups for activity and was unaffected by reagents that react with carboxyl groups.

Keywords. Proteases; finger millet; *Eleusine coracana*; trypsin/amylase inhibitor.

Introduction

Plant protease inhibitors and amylase inhibitors which are proteinaceous in nature are known to disappear during the process of germination (Ryan, 1973; Chrispeels and Baumgartner, 1978; Shivaraj and Pattabiraman, 1980). Veerabhadrappa *et al.* (1978) first showed that antitryptic activity and antichymotryptic activity were markedly reduced in the endosperm of finger millet on germination. This can be attributed to the proteolytic cleavage of the inhibitory proteins during germination. Characterization of many proteases from germinated cereals and legume seeds has been accomplished using exogenous as well as endogenous substrates (Ashton, 1976; Baumgartner and Chrispeels, 1977; Fujimaki *et al.*, 1977). However, little information is available on the action of seed proteases on the endogenous protease inhibitors. An earlier communication from this laboratory reported the isolation and characterization of an inhibitor from finger millet acting on both trypsin and α-amylase (Shivaraj and Pattabiraman, 1981). Using this factor as a substrate, the proteolytic activity of finger millet during germination was studied and the results were compared with the proteolytic activities observed with haemoglobin and albumin as substrates.

Materials and methods

Chemicals

Hog pancreatic amylase (EC 3.2.1.1, type III A, twice crystallised), Pepstatin, bovine haemoglobin, α-N-benzoyl DL-arginine *p*-nitroanilide (BAPNA) and dia-

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Abbreviations used: BAPNA, α-N-Benzoyl-DL-arginine *p*-nitroanilide; DON, diazoacetyl DL-norleucine methyl ester; DTNB, 5,5′-dithiobis nitrobenzoic acid; TAI, trypsin/amylase inhibitor.

zoacetyl DL-norleucine methyl ester (DON) were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Bovine trypsin (EC 3.4.21.4, salt free and twice crystallized) was the product of Worthington Biochemical Corporation, Freehold, New Jersey, USA. -Dithiobis 2-nitro-benzoic acid (DTNB) was procured from Pierce Chemicals, Rockford, Illinois, USA. All other reagents were of analytical grade.

Germination of finger millet

This was carried out in batches. Two g of the grain was soaked in a wet cloth for 16 h and then allowed to germinate in a petri dish under diffused light at room temperature (25–28° C). At the end of 2, 3, 4, 5 and 6 days of germination, the seedlings were extracted with 20 ml of water at 4° C. The extract was centrifuged at 5 000 *g* for 10 min in a Remi C-24 centrifuge at 4° C. The supernatant was used as the source of proteases.

Isolation of trypsin/amylase inhibitor (TAI)

This was done by the conventional purification method (Shivaraj and Pattabiraman, 1980) and also by affinity chromatography (Shivaraj *et al*, 1982). TAI prepared by both the methods behaved similarly when used as substrates for finger millet proteases. Hence, in all subsequent studies the inhibitor isolated by the affinity chromatographic method was employed.

Assay of proteolytic activity of germinated finger millet extract using TAI as substrate

An aliquot (20–100 ul) of the extract was incubated with 5.6 μg protein of TAI at 37° C in presence of 50 μl of 0.5% 2-mercapto-ethanol and 50 μl of 0.05 *M* buffers of varying pH (HCl-KCl, pH 1.5–2.0; glycine-HCl, pH 2.5–3.5; acetate, pH 4.0–5.0; sodium phosphate, pH 6.0 and Tris-HCl, pH 8.0) in a total volume of 0.2 ml for 3 h. To assay the residual antitryptic activity of TAI, trypsin solution (10–12 μg) was added to the incubation mixture followed by 2.0 μmol of BAPNA and 100 μmol of phosphate buffer, pH 7.6 to a final volume of 3.0 ml. After 30 min incubation at 37° C, the tryptic activity was terminated by the addition of 2.0 ml of 30% acetic acid (v/v). *p*-Nitro-aniline liberated was measured at 410 nm. Controls with heat treated (100° C, 2 min) extract of finger millet were run simultaneously.

To measure the residual antiamylase activity of TAI after the action of the proteases, 0.1 ml of the incubation mixture (see above) was treated with hog pancreatic amylase (0.3 μg protein) at 37° C for 20 min in presence of 60 μmol of sodium phosphate buffer, pH 6.9 containing 10.5 μmol of NaCl (total volume 1.5 ml). The amylase action was initiated by the addition of 0.5 ml of 1% starch solution. After 5 min incubation at 37° C, the reaction was arrested with 1 ml of dinitrosalicylate reagent and processed further as described earlier (Shivaraj and Pattabiraman, 1980). Controls were performed during the assay with heat treated finger millet extract.

One unit of tryptic activity was the amount that liberated 1 μmol of *p*-nitroaniline under the assay conditions. One unit of amylase activity was equivalent to the amount of amylase that liberated 1 μmol of reducing sugar (maltose equivalent) under the assay conditions. One unit of inhibitor, is the amount that decreased the tryptic (or amylase) activity by one unit.

Protease activity of germinated finger millet extract with haemoglobin or albumin as substrate

The extract (0.2 ml) was incubated with 20 mg of denatured haemoglobin (or albumin) in a volume of 2.5 ml in presence of 50 µmol of buffers of varying pH values (pH 2.5-8.0, see above). After 4 h incubation at 37° C, the reaction was terminated by the addition of 1.5 ml of 10% trichloroacetic acid (w/v). After 30 min, the precipitate formed was sedimented by centrifugation at 2500 *g* for 10 min and 1 ml of the clear supernatant was analyzed for the soluble peptides by the method of Lowry *et al.* (1951). One unit of proteolytic activity is equal to the amount that formed 1 mg of trichloroacetic acid soluble fragments under the assay conditions.

Protein estimation

Protein content of the finger millet extracts was measured by the dye binding method using Coomassie Brilliant Blue-G (Read and Northcote, 1981).

Results

The effect of the trypsin/amylase inhibitor isolated from dormant finger millet was tested for its action on the protease activity of germinated finger millet extracts at different pH values in the range 2.5–7.0. Haemoglobin was used as substrate in these studies. It was found that the inhibitor did not suppress the endogenous protease activity. Similarly TAI had no effect on the endogenous amylase activity of the millet extract when assayed at pH 6.9. These data indicate the possibility of the proteolytic digestion of TAI by the endogenous proteases in the germinated millet extract. When TAI was incubated with the seed extract and then tested for antitryptic and antiamylase activities, against bovine, trypsin, and hog pancreatic amylase respectively, there was a decrease of inhibitory potency. It was found that there was proportional loss of trypsin inhibitory activity and amylase inhibitory activity when TAI was treated with germinated millet extract. The data are shown in table 1. These results provide additional evidence for the earlier observation, that antiamylase and antitryptic activities reside in the same protein (Shivaraj and Pattabiraman, 1981).

The proteolytic activity was found to be maximum in 3 days germinated finger millet extracts when estimated with either TAI or haemoglobin as substrates. The activity profiles at different stages of germination are depicted in figure 1. The results are based on the calculation of proteolytic activity at pH 2.5 for TAI substrate and at pH 5.0 with haemoglobin as substrate, since these were the optimum pH values required for the corresponding substrates (figure 2).

The protease activity responsible for the inactivation of TAI, required SH-groups for maximal activity. Omission of 2-mercaptoethanol in the assay system at pH 2.5 led to a 65% reduction in enzyme activity. Inclusion of DTNB (500 µg) in the assay system completely abolished the enzyme activity. On the other hand, at pH 5.0 with haemoglobin as substrate, the proteolytic activity was not dependent on SH-groups. Addition of 2-mercaptoethanol did not increase the proteolytic activity at pH 5.0. Further, treatment of finger millet extract with DTNB (500 µg) both at pH 5.0 and at pH 7.5 did not cause any change in haemoglobin cleaving activity.

Table 1. Action of proteases of 3-day germinated finger millet extract on the antitryptic and antiamylase activities of TAI.

pH	Residual activity (%) Antitryptic	Antiamylase
2.5	39	32
4.0	50	42
6.0	62	57
8.0	80	76
Control	100	100

TAI was incubated with finger millet extract for 3 h at 37° C at different pH values and residual inhibitory activities were determined. Other details are given under 'materials and methods'.

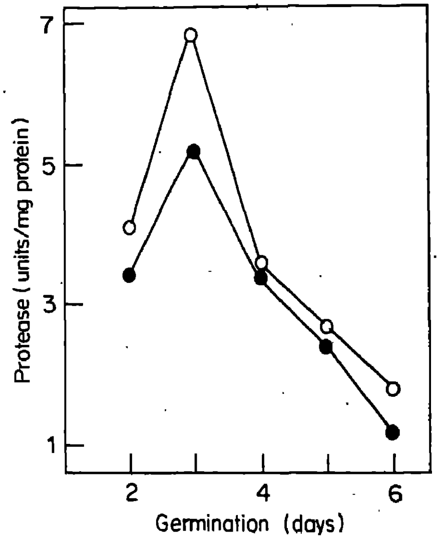

Figure 1. Proteolytic activity of germinated finger millet extract at different days of germination. Using TAI as substrate at pH 2.5, (○). The protease activity was measured on the basis of disappearance of antitryptic activity of the inhibitor. Using haemoglobin as substrate at pH 5.0, (●).

Addition of DON (250 μg) or Pepstatin (250 μg) to the germinated finger millet extract abolished the protease action on TAI. On the other hand, these modifiers did not affect the haemoglobin splitting activity at pH 5.0. The results are shown in table 2. These data suggest that the protease that acts on TAI is a carboxyl proteinase.

The protease that inactivates TAI was found to be highly labile on storage. The activity disappeared completely on standing of the millet extract at 4° C for 72 h. In contrast, 65% of the original haemoglobin splitting activity at pH 5.0 remained on storage of the extract at 4 ° C for 6 days. The relative stabilities of the two activities

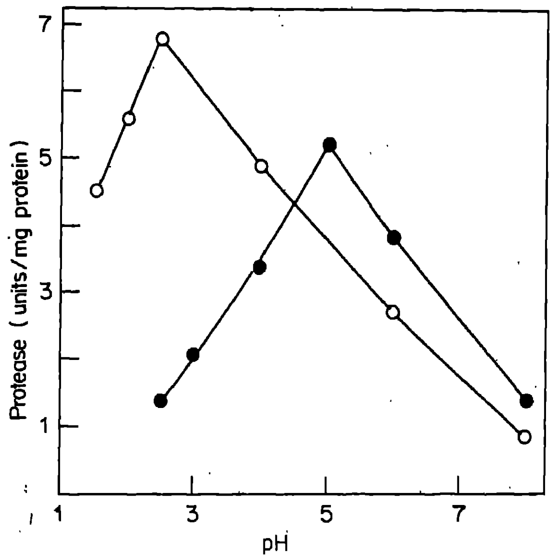

Figure 2. Proteolytic activity of 3 day germinated ragi extract at different pH values. Using TAI as substrate, (○). The protease activity was measured on the basis of disappearance of antitryptic activity of the inhibitor. Using haemoglobin as substrate, (●)

Table 2. Action of carboxyl group modifiers on the finger millet proteases.

Modifying agent	Protease activity (%)	
	TAI as substrate*	Haemoglobin substrate
None	100	100
DON (250 μg)	0	100
Pepstatin (250 μg)	5	100

*The protease activity was measured in terms of disappearance of antitryptic activity of TAI.

are shown in figure 3. When the proteolytic activity in germinated finger millet extracts was measured with albumin as substrate, the pH optimum was 3.5. However, the activity with albumin as substrate was only 25% compared to the activity with haemoglobin as substrate.

Discussion

The present studies show that there are at least two different proteases in germinating finger millet, one that has an optimum pH 2.5 and the other with an optimum pH of 5.0. The latter enzyme was relatively more stable and was not dependent on SH-groups for activity. The former enzyme was relatively labile and required SH-groups for maximal activity. Sulphydryl dependent acid proteases have been isolated and characterized during germination from barley (Burger, 1973), mung bean (Baumgartner and Chrispeels, 1977), kidney bean (Vavreinova and Turkova, 1975) and corn (Abe *et al*, 1978). The corn enzyme is reported to have the lowest pH optimum of 3.0 for all the sulphydryl proteases known. The

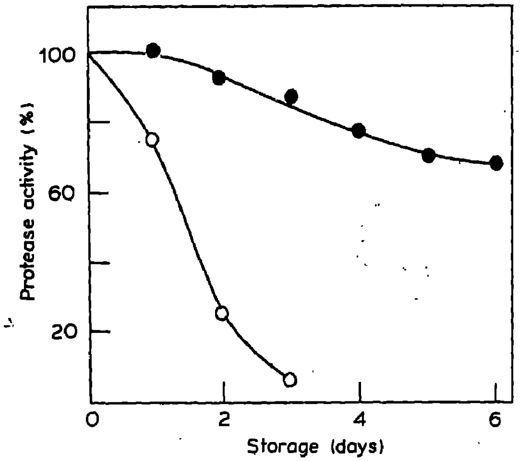

Figure 3. Effect of storage of 3 day germinated ragi extract at 4° C on the endogenous protease activity. Using TAI as substrate at pH 2.5, (○). The protease activity was measured on the basis of disappearance of antitryptic activity of the inhibitor. Using haemoglobin as substrate at pH 5.0, (●).

acid protease in finger millet reported here has a pH optimum lower than that. The active site of the enzyme probably has a carboxyl group since Pepstatin and DON inhibited its action. The SH-groups may be essential for the conformational stability of the enzyme. Further studies with purified enzymes are needed to confirm this suggestion. To the best of our knowledge, this report is the first that demonstrates the presence of a protease that needs a carboxyl group for activity in germinating plant seeds. It is probable that many of the sulphydryl dependent acid proteases reported in the plant systems could in fact be carboxyl proteases requiring SH-groups for stability.

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Sodium transport in filamentous nitrogen fixing cyanobacteria

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Abstract. Two filamentous, nitrogen fixing cyanobacteria were examined for their salt tolerance and sodium (Na^+) transport. *Anabaena torulosa*, a saline form, grew efficiently and fixed nitrogen even at 150 mM salt (NaCl) concentration while, *Anabaena* L-31, a fresh water cyanobacterium, failed to grow beyond 35 mM NaCl. *Anabaena torulosa* showed a rapidly saturating kinetics of Na^+ transport with a high affinity for Na^+ (K_m, 0.3 mM). *Anabaena* L-31 had a much lower affinity for Na^+ (K_m, 2.8 mM) than *Anabaena torulosa* and the pattern of uptake was somewhat different. Both *Anabaena* spp. exhibited an active Na^+ extrusion which seems to be mediated by a Na^+-K^+ ATPase and aided by oxidative phosphorylation. *Anabaena* L-31 was found to retain much more intracellular Na^+ than *Anabaena torulosa*. The results suggest that the saline form tolerates high Na^+ concentrations by curtailing its influx and also by an efficient Na^+ extrusion, although these alone may not entirely account for its success in saline environment.

Keywords. Nitrogen fixation; cyanobacteria; sodium transport; salt tolerance.

Introduction

Sodium is the predominant soluble cation in most saline soils and waters. Although cyanobacteria are known to require Na^+ for growth (Allen and Arnon, 1955; Kratz and Myers, 1955) and nitrogen-fixation (Apte and Thomas, 1980) the physiological processes involved in their salt-tolerance are not known. Ion transport in cyanobacteria is a generally neglected phenomenon, and Na^+ transport in particular has been studied only in the unicellular cyanobacterium *Anacystis nidulans*. In this organism, Na^+ transport has been shown to be regulated by an active extrusion of the cation by a proton antiport system (Dewar and Barber, 1973; Paschinger, 1977), a mechanism characteristic of certain bacteria (Rothstein, 1972) and sensitive to energy transfer inhibitors (Paschinger, 1977). In an earlier report (Apte and Thomas, 1974) we have shown the ability of a fresh water cyanobacterium *Anabaena* L-31 and a saline form *Anabaena torulosa* to absorb Na^+. In this paper, we describe the details of the kinetics of Na^+ transport in these cyanobacteria as revealed by the use of radiotracer $^{22}Na^+$. The results suggest that the Na^+ transport properties of cyanobacteria from saline habitats are better suited for their survival in such environments.

Abbreviations used: DCMU, 3-(3,4-Dichlorophenyl)-1,1-dimethylurea; CCP, carbonyl cyanide, *m*-chlorophenyl hydrazone; DNP, 2,4-dinitrophenol; DCCD, N,N′-dicyclohexylcarbodiimide; BBOT, 2,5-(5-ditetrabutyl-2-benzoxazolyl)-thiophene; ATPase, Adenosine triphosphatase.

Materials and methods

Microorganisms

Two filamentous, N_2-fixing cyanobacteria *Anabaena torulosa* (Apte and Thomas, 1980) and *Anabaena* L-31 (Thomas, 1970), were used in axenic condition. *Anabaena* L-31 was isolated from a paddy field and is a fresh water inhabitant. *A. torulosa* was isolated from a brackish soil and is known to be characteristic of saline waters (Desikachary, 1959).

Culture conditions and growth

Cyanophycean medium (David and Thomas, 1979), diluted five-fold and free of combined nitrogen, was used for the maintenance of all the cultures. Cyanobacteria were grown under constant aeration (2 litre/min) at 5000 lux and 25° C. Salt tolerance of cyanobacteria was examined on agar slants (1% agar, w/v) containing various concentrations of NaCl. Chlorophyll *a* determined as described by Mackinney (1941) was used as index of growth. In long term experiments (6 days) as the cell density increased, the distribution of light became uneven, and chlorophyll *a* concentration could not serve as a satisfactory index of growth. In such experiments, increase in total N, determined by micro-kjeldahl analysis (Steyermark, 1961) was preferred. Nitrogenase activity assays were performed as described previously (David *et al.*, 1980).

Uptake of sodium

Sodium uptake was determined by using the radiotracer ^{22}Na. Logarithmic phase cultures were harvested, washed and inoculated in N-free cyanophycean medium without Na^+. After incubation for 24 h, to induce Na^+ deficiency, filaments were filtered, washed and resuspended in cyanophycean medium (without Na^+) and used in experiments. Test suspensions, unless stated otherwise, contained ^{22}NaCl (0.25 μCi/ml) mixed with non-radioactive NaCl to give a final concentration of 0.5 mM or 10.0 mM Na^+. Assays involving incubation for more than 5 min were performed under aeration (2 litre/min) at 5000 lux and 25° C. Short term incubations were carried out in 5 ml vials held on a rotary wheel (11 r.p.m.), light intensity and temperature remaining the same as above. At the end of a desired period, 2 ml suspension was filtered on a Whatman GF/C paper circle (Whatman Ltd., UK) and the residue was washed with 100 ml of equimolar unlabelled NaCl to completely remove the diffusible and easily exchangeable fraction of Na^+ (nearly 90% of the radioactivity initially bound to cells) followed by washing with distilled water. Filter paper discs with samples were dried, transferred to scintillation vials containing 10.0 ml 2,5-(ditetrabutyl-2-benzoxazyolyl)-thiophene (BBOT) (0.4% w/v in toluene : methanol, 1 : 1) and counted in a Beckman LS-100 C liquid scintillation spectrometer (Beckman Instruments Inc., California, USA). The kinetic data were evaluated by standard linear regression analysis for calculating kinetic constants.

Chemicals

All inorganic salts were obtained from Sarabhai M. Chemicals, Baroda, or British Drug Houses, Bombay. ^{22}NaCl was acquired from Radiochemical Centre, Amersham, UK and bacto-agar from Difco Laboratories, Michigan, USA. 3-(3,4- Dichlorophenyl)-1,1-dimethylurea (DCMU), 2,4-dinitrophenol (DNP), carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), N,N′-dicyclohexyl carbodiimide (DCCD), Ouabain and BBOT were supplied by Sigma Chemicals Co., St. Louis, Missouri, USA. Concanavalin A was obtained from Calbiochem, San Diego, California, USA. Acetylene was supplied by Indian Oxygen Ltd., Bombay, and pure ethylene from Matheson Gas Products, New Jersey, USA.

Results

Salt-tolerance

Figure 1 shows the growth and nitrogenase activity of cyanobacteria in the presence of different concentrations of NaCl. *Anabaena torulosa* was fairly salt-tolerant growing even at 150 mM NaCl. The salt concentration responsible for 50% inhibition was found to be 115 mM for growth and 150 mM for nitrogenase activity. Significantly at 2–35 mM NaCl concentrations, *A. torulosa* showed better growth and

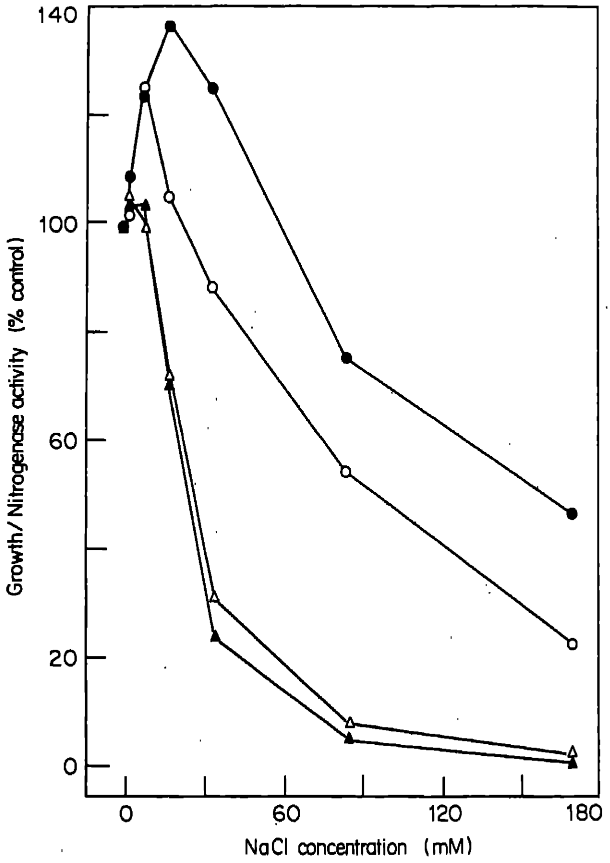

Figure 1. Salt tolerance of nitrogen fixing cyanobacteria: *Anabaena torulosa* (○, ●); *Anabaena* L-31 (△, ▲). Growth (chlorophyll *a*) (open symbols) and nitrogenase activity (C_2H_2 reduction) (closed symbols) were measured 5 days after inoculation. See text for details.

higher nitrogenase activity than in control experiments without salt. In contrast, similar salt concentrations did not stimulate growth or nitrogenase activity in *Anabaena* L-31 and the cyanobacterium was found to be very sensitive to salt, not growing beyond 35 mM NaCl. Salt concentration of 25 mM caused 50% inhibition of growth and nitrogenase activity in this cyanobacterium.

Kinetics of sodium transport

There was no lag in the uptake of Na^+ which stabilised within a short time in both the cyanobacteria. But the rates and pattern of uptake differed (figures 2, 3). In *A. torulosa* the initial exponential phase of uptake lasted only for 1 min and stabilised quickly at both 0.5 mM and 10.0 mM Na^+ concentrations. At lower concentration of Na^+ (0.5 mM) the cyanobacterium showed higher rates of uptake than

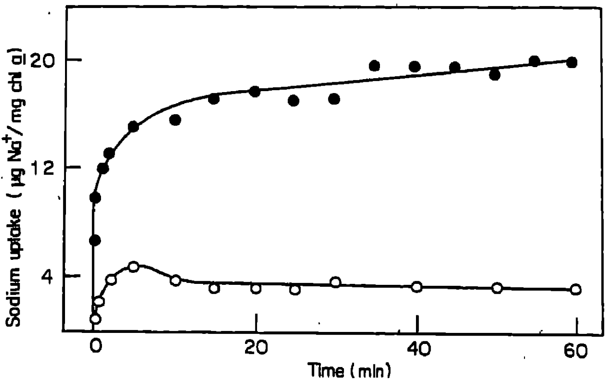

Figure 2. Kinetics of Na^+ uptake by *Anabaena torulosa* at 0.5 mM (○) and 10.0 mM (●) external Na^+ concentrations.

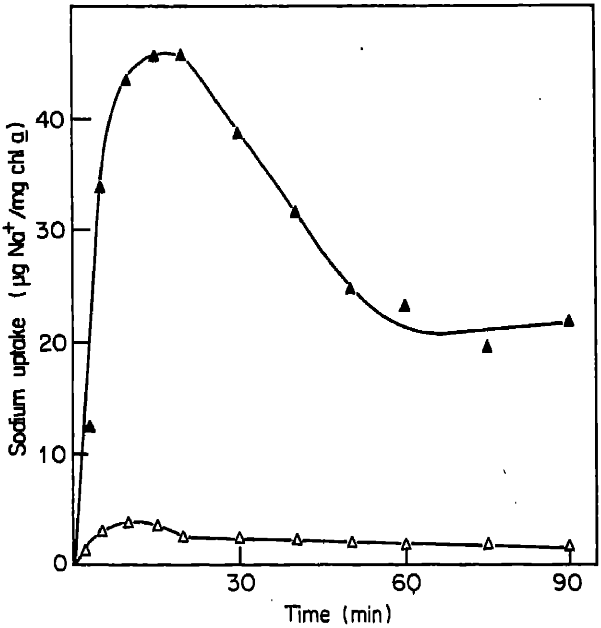

Figure 3. Kinetics of Na^+ uptake by *Anabaena* L-31 at 0.5 mM (△) and 10.0 mM (▲) external Na^+ concentrations.

Anabaena L-31. At higher Na^+ concentration (10 mM) the initial uptake was much more rapid in *Anabaena* L-31 than in *A. torulosa* but was followed by an extrusion of Na^+ till 60 min when it stabilised.

Long term experiments showed that in both cyanobacteria maximum Na^+ accumulation was attained within first few hours and then a lower level was maintained for a prolonged period of 6 days (figure 4). *Anabaena* L-31 retained 2.7 times more Na^+ than *A. torulosa* at the end of six days, the values being 9.6 μg Na^+/mg N and 3.5 μg Na^+/mg N respectively. Similar trend was observed in the intracellular concentrations of Na^+ in these cyanobacteria. After 18 h of equilibration in the medium containing $^{22}Na^+$ the internal concentrations of Na^+ were estimated on the basis of radioactivity per ml packed cell volume. Values of 97 μM and 0.36 mM for *A. torulosa* and 60 μM and 0.51 mM for *Anabaena* L-31 were observed at 1 and 10 mM Na^+ in the external medium.

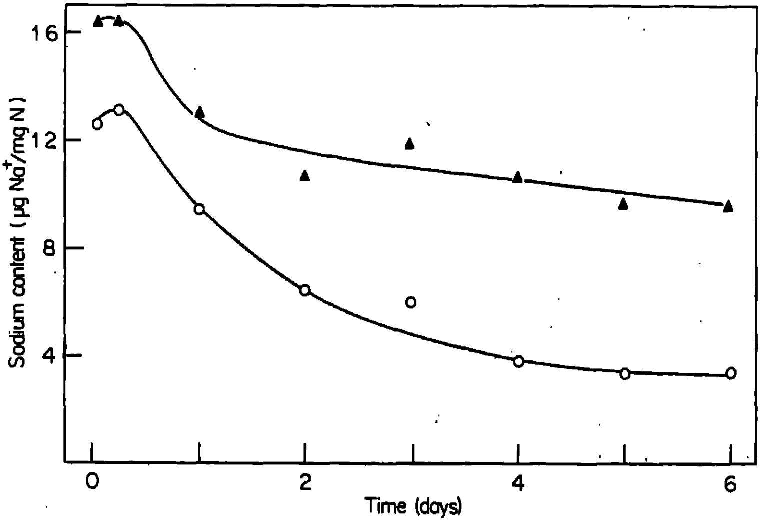

Figure 4. Retention of sodium by *Anabaena torulosa* (O) and *Anabaena* L-31 (Δ) during growth under N_2 fixing conditions. Increase in total N was chosen as the measure of growth in this experiment for reasons specified in the Materials and methods.

In the presence of increasing external concentrations of Na^+, uptake of Na^+ in both cyanobacteria followed Michaelis-Menten kinetics saturating between 10–20 mM (figures 5, 6). Michaelis constants (K_m) calculated from the double reciprocal plots (figures 5, 6 insects) were 0.3 mM for *A. torulosa* and 2.8 mM for *Anabaena* L-31. These values are in conformity with the observed differences in the rates of Na^+ uptake by these cyanobacteria at different Na^+ concentrations.

Effect of metabolic inhibitors on Na^+ uptake

Light was found to facilitate Na^+ uptake in both cyanobacteria and preincubation in dark resulted in reduced Na^+ uptake (table 1). Addition of inhibitors of photosynthetic electron transport (DCMU) or photophosphorylation (CCCP) was not very effective at concentrations (1 μM) which inhibited photosynthesis (data not shown) but Na^+ uptake was inhibited at higher concentrations (10 μM) (table 1). DNP, an inhibitor of oxidative phosphorylation, enhanced Na^+ uptake in both

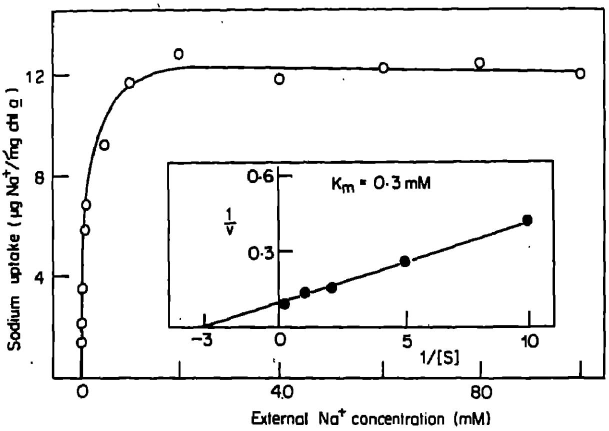

Figure 5. Effect of external Na$^+$ concentration on Na$^+$ uptake by *Anabaena torulosa*. Duration of assay was 15 s. No change in cell size or packed cell volume was noticed at any concentration of Na$^+$. Inset shows a double reciprocal plot of the data for calculation of Michaelis constant (K_m).

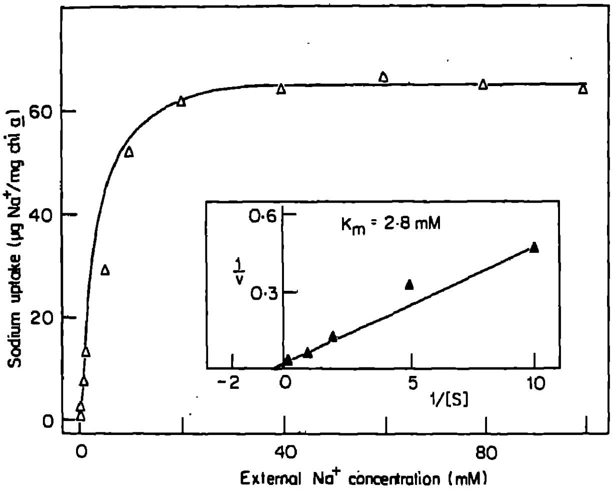

Figure 6. Effect of external Na$^+$ concentration on Na$^+$uptake by *Anabaena* L-31. Duration of assay was 2 min. No change in cell size or packed cell volume was noticed at any concentration of Na$^+$. Insert shows a double reciprocal plot of the data for calculation of Michaelis constant (K_m).

cyanobacteria. Significant enhancement of Na$^+$ uptake was noted in the presence of ouabain, a specific inhibitor of Na$^+$-K$^+$ ATPase, while DCCD, an inhibitor of classical ATPase, was not effective or marginally inhibited the uptake (table 1) at concentrations which were adequate to inhibit ATPase (Paschinger, 1977). Concanavalin A showed a differential effect on Na$^+$ uptake in these cyanobacteria. While Na$^+$ uptake was enhanced in *A. torulosa* by treatment with concanavalin A, in *Anabaena* L-31 it was inhibited. Gramicidin stimulated Na$^+$ uptake both in light as well as in dark (table 1).

Table 1. Effect of various inhibitors* on Na$^+$ uptake by N$_2$-fixing cyanobacteria.

Inhibitor (concentration μM)	Radioactivity/ml (% control)	
	Anabaena torulosa	*Anabaena* L-31
Control	100	100
Dark	74	79
DCMU		
(10)	140	125
(10)	61	78
CCCP		
(1)	100	99
(10)	98	65
(100)	70	45
DNP		
(10)	162	131
(100)	297	162
DCCD		
(50)	100	92
(200)	90	79
Ouabain		
(10)	135	109
(100)	192	159
Concanavalin A		
(50 μg/ml)	132	100
(200 μg/ml)	186	65
(400 μg/ml)	120	52
Gramicidin (light)		
(5 μg/ml)	130	139
(20 μg/ml)	172	225
Gramicidin (dark)		
(20 μg/ml)	122	143

* Prior to assay cyanobacterial suspensions were pretreated with the inhibitor for 15 min. Dark treatment (No. 2 and 10) consisted of preincubation of aerated cultures in dark for 5 h followed by assays also performed in the dark. The assays were initiated at time zero by addition of 0.25μCi of ^{22}Na$^+$ and stable Na$^+$ at a final concentration of 10.0 mM in 1.0 ml final volume. Duration of assay was 15 s for *A. torulosa* and 2 min for *Anabaena* L-31, when Na$^+$ uptake was in the linear phase.

Discussion

Na$^+$, an important nutritional requirement in cyanobacteria (Allen and Arnon, 1955; Kratz and Myers, 1955) has recently been shown to be essential for nitrogenase activity in *Anabaena* L-31 and *A. torulosa* (Apte and Thomas, 1980). In the present study these cyanobacteria have been found to differ considerably in their ability to resist salt-stress (figure 1). *A. torulosa* exhibits good growth and nitrogenase activity at salt concentrations well beyond the normal tolerance limit of many microbes and crop plants. On the other hand *Anabaena* L-31, a fresh water form, fails to grow beyond 35 mM NaCl. The difference in salt-tolerance seems to be related to the Na$^+$ transport pattern of these cyanobacteria. In *A. torulosa* Na$^+$ uptake saturates quickly (figure 2), follows a Michaelis-Menten kinetics and shows a high affinity for Na^+ (figure 5). *Anabaena* L-31 also shows a Michaelis-Menten type of rapid uptake (figure 3) but it has a much lower affinity for Na^+ (figure 6) than *A. torulosa*. The difference in K_m is probably in accordance with the metabolic requirement of Na^+ in a brackish and a fresh water form. Concentrations of Na^+ required for optimum growth are 0.5 mM for *Anabaena* L-31 (Apte and Thomas, unpublished results) and nearly 10 mM for *A. torulosa* (figure 1).

In *A. torulosa* and *Anabaena* L-31, Na^+ influx appears to be affected by light mediated metabolism since dark incubation inhibits Na^+ uptake. However this effect is not consequent to the inhibition of photosynthesis since concentrations of DCMU and CCCP required to inhibit Na^+ uptake are several times higher than those required to inhibit photosynthesis (table 1). Gramicidin, known for its stimulatory effect on membrane permeability, stimulates the effect of light and increases Na^+ uptake in dark. Although the absolute quantity of Na^+ taken up is more in light, the per cent enhancement of Na^+ uptake caused by gramicidin in light and dark is comparable. This suggests that light may be involved in maintaining membrane permeability suitable for the transport of this cation and the greater effect of gramicidin in light may therefore be due to additive increase in permeability. In *A. nidulans* also light was found to non-specifically increase the permeability of plasmamembrane (Paschinger, 1977).

Na^+ influx in *A. torulosa* and *Anabaena* L-31 is probably a passive carrier mediated diffusion process while the regulation of Na^+ transport is achieved by an active extrusion of Na^+. The Na^+ extrusion appears to be mediated by an ouabain sensitive Na^+–K^+ ATPase which is distinct from the conventional F_1F_0 ATPase in being insensitive to DCCD (Paschinger, 1977; Heefner and Harold, 1982). This contrasts with the situation in *A. nidulans* where an active Na^+ efflux is driven by a Na^+/ H^+ antiporter involving a proton translocating ATPase sensitive to DCCD and CCCP (Paschinger, 1977), and resembles that in *Streptococcus faecalis*, where efflux is mediated by a Na^+ stimulated ATPase which is insensitive to several inhibitors of conventional ATPase (Heefner and Harold, 1982). However in *S. faecalis* the ATP driven Na^+ pump requires only ATP but does not require proton motive force since reagents which collapse proton motive force (DCCD, CCCP, valinomycin) are ineffective. In *Anabaena* spp., although DCCD and CCCP are ineffective, Na^+ efflux probably requires a H^+ gradient, since gramicidin which destroys the proton motive force also inhibits efflux (table 1). The "Na^+ pump" of *Anabaena* spp. can therefore be described as a Na^+/H^+ or Na^+/K^+ antiporter similar to Na^+/H^+ antiporter in *A. nidulans* but involving an ouabain sensitive ATPase distinct from the conventional ATPase. The energy source for Na^+ extrusion may be ATP derived from oxidative phosphorylation since DNP inhibits efflux. Both ouabain and DNP cause a more dramatic effect on the efflux in *A. torulosa* indicating that it has a more efficient Na extruding system than *Anabaena* L-31.

Concanavalin A has been shown to inhibit mitochondrial ATPase in yeast (Satav *et al*, 1980) by binding to the F_1 portion which is said to be a glycoprotein. The observed effect of concanavalin A on Na^+ uptake in *A. torulosa* however may not be through its effect on ATPase since DCCD is ineffective. The effect, which is more likely to be due to the ability of concanavalin A to bind certain sugar receptors (polysaccharides/glycoproteins) on plasmamembrane and to reorient them, needs further investigation.

Anabaena L-31 retains much more Na^+ than *A. torulosa* (figure 4) probably in excess of its requirement as shown by the lack of correlation of sodium concentration with growth or nitrogenase activity (figure 1). Apparently this Na^+ accumulation is not a physiological adaptation but arises out of a failure to regulate its intracellular content of Na^+ and results in its lack of survival under salt stress. *A. torulosa* achieves this regulation by curtailing the uptake itself and also by more efficient extrusion of Na^+ than *Anabaena* L-31. Salt-tolerance of *A. torulosa* resembles those of glycophytes rather than halophytes (Flowers *et al*, 1977). It is known that organic acids, amino acids or carbohydrates accumulate under stress to build-up the osmotic potential (Flowers *et al*, 1977). We have not studied this aspect but the enhanced nitrogenase activity in *A. torulosa* under salt stress may be aimed at eventual accumulation of amino acids for better osmoregulation. Additionally, the ability of *Anabaena torulosa* to sporulate (Fernandes and Thomas, 1982), a feature with high adaptive value, and to form mats (which probably create microenvironments with decreased salinity) would contribute to the success of this cyanobacterium in saline environment.

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An *in vitro* system to study drug sensitivity of *Mycobacterium leprae* using infected human tissue

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Abstract. A reliable screening technique for assessing the sensitivity of *Mycobacterium leprae* to drugs has been developed. The method is based on the susceptibility or otherwise of *M. leprae*—infected tissues from lepromatous leprosy patients to the action of diaminodiphenyl sulphone (dapsone) or rifampicin on the incorporation of [^{14}C]-acetate into lipids. The extent of inhibition or lack of inhibition correlated very well with the drug sensitivity or resistance of the bacteria isolated from the patients to the above drugs. A similar trend was observed when the incorporation into individual fractions of neutral lipids was measured. There was no incorporation by heat-killed tissues. This method correlates well with the 3,4-dihydroxyphenylalanine uptake studies.

Keywords. Drug sensitivity/resistance; *Mycobacterium leprae*; [^{14}C]-acetate uptake.

Introduction

Successful development of antimicrobial compounds is the end result of a rational screening programme against known microbes by simple and quick *in vitro* systems, followed by preliminary toxicological studies on the compounds which show promise. All microbes that can be cultivated can be subjected to these screening tests. *Mycobacterium leprae* which has resisted *in vitro* cultivation despite efforts by several workers, cannot be tested by these methods. The mouse foot pad model of Shepard (1960) and Rees (1964) is the most widely used method, but is limited by the long time period of 6 to 9 months required for assay.

There are currently two *in vitro* tests in use, one developed in our laboratory by Ambrose *et al.* (1974, 1978) using uptake of labelled L-3, 4-dihydroxy (ring-2,5,6-^{3}H) phenylalanine (DOPA) by partially purified *M. leprae* and the other using incorporation of [^{3}H]-thymidine by *M. leprae* inside the macrophages (Drutz and Cline, 1972; Talwar *et al.*, 1974; Prasad and Nath, 1981; Nath *et al.*, 1982).

Initial studies in our laboratory showed that *Mycobacterium lepraemurium* infected liver and spleen tissue from mice, when incubated with [^{14}C]-acetate for one week in Minimal Eagle's medium containing serum, incorporated the precursor into the bacterial-bound lipid portion, identified as mycolic acids. The incorporation decreased in the presence of drugs like isoniazid, rifampicin and streptomycin. These observations formed the basis to study the incorporation of [^{14}C]-acetate into the lipids of *M. leprae* in human biopsy material, freshly obtained from patients, in the presence and absence of known antileprosy drugs namely

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Abbreviations used: DOPA, L-3,4-Dihydroxyphenylalanine; dapsone, diaminodiphenyl sulphone.

diaminodiphenyl sulphone (dapsone) and rifampicin. In this paper, we describ the effect of dapsone and rifampicin on the incorporation of $[^{14}C]$-acetate in experiments to study the synthesis of the various lipid components by *M. lepr* contained in biopsy tissue obtained from leprosy patients and the effect of dapso and rifampicin.

Materials and methods

Uptake of $[^{14}C]$-acetate

Human skin biopsis from *M. leprae* infected patients were obtained from th Acworth Leprosy Hospital, Bombay. The biopsies were collected from lepromat ous leprosy patients from the ear lobe or young skin lesions, under asceptic condi tions. It was processed within 3 h after collection. The tissue was washed wit saline followed by a wash with Minimal Essential Medium containing penicilli (50 units/ml). It was chopped into small pieces and each piece was tranasferred t a 10 ml flask containing Eagle's Minimal Essential Medium, containing addition 20% AB type human serum.

Two controls were used, one untreated tissue and the other heat-killed tissu The drug-treated tissues included dapsone (10 μg/ml) and rifampicin (5 μg/ml The reaction mixture contained 0.5 μCi/ml of 1,2-$[^{14}C]$-acetate (specific activit 58.6 mCi/mmol; obtained from Isotope Division, Bhabha Atomic Researc Centre, Bombay). Penicillin (100 units/ml) and Mycostatin (50 units/ml) wer added to the medium as antibacterial and antifungal agents. The cultures wer incubated at 34±1° C and the flasks were kept in a dessicator with 100% humidit and maintained for 11 days, the medium containing $[^{14}C]$-acetate was change every 3 days.

On completion of this period, the tissue was suspended in about 3 ml of salin and autoclaved at 15 lbs pressure for 30 min. The tissue was homogenized in power driven Potter-Elvehjem type homogenizer fitted with a teflon pestle for min and the homogenate was centrifuged at 182 *g* in a Sorvall refrigerated centri- fuge to remove the tissue debris. The supernatant, containing the bacilli was cen- trifuged at 11,700 *g* for 15–20 min. The sediment, containing bacilli was washe with saline and after recentrifugation, suspended in an appropriate volume o saline. Bacillary lipids were extracted by the method of Folch *et al.* (1957) Radioactivity in each preparation at various stages was determined using Kontron MR 300 Liquid Scintillation spectrometer after mixing the sample wit Bray's scintillation fluid (Bray, 1960).

The extracted lipids were separated by thin layer chromatography using Silica Gel G (BDH) (0.25 mn thickness) and a solvent system containing hexane : ether : glacial acetic acid (80 : 20 : 1 v/v). The separated neutral lipids were visualised with iodine and identified by comparison with standards. The radioactivity associated with the various lipids was measured by scrapping the gel corresponding to the standard spots from the plate and suspending in Bray's scintillation fluid and measuring the radioactivity. Normal skin biopsies obtained from non-leprosy individuals was also processed in a similar manner. All reagents used were of the analytical grade.

Uptake of $[^{3}H]$-DOPA

Eight biopsies were processed for drug trials by the method described above and by the method developed earlier in our laboratory using L-3,4-dihydroxy (ring-2,5,6-^{3}H) phenylalanine (specific activity 1 Ci/mmol; obtained from Amersham, England) uptake as an index of bacterial viability and drug sensitivity of *M. leprae* (Ambrose *et al.*, 1974, 1978).

Results

Effect of drug on [^{14}C]-acetate incorporation in normal tissues

Before one could study the uptake of [^{14}C]-acetate in lepromatous tissue one should know the type of incorporation of the precursor into lipids of normal skin. The uptake of [^{14}C]-acetate by the normal skin tissue and its incorporation into lipids in the presence and absence of dapsone is shown in table 1. The relative incorporation in the lipids under both the experimental condtions was similar suggesting that dapsone had no significant effect on the lipid synthesis of cells in normal skin tissue.

Table 1. Relative incorporation of [^{14}C]-acetate into lipids of normal skin in presence and absence of dapsone.

	Incorporation of acetate into lipids (%)[a]	
Biopsy No.	Control	Dapsone (10 μg/ml)
1	35	44
2	50	53
3	16	16
4	15	18
5	20	29
	27[b]±6 (mean ±SE)	32[b]±4

[a] Results expressed as percentage of radioactivity incorporated into lipids in relation to total uptake of the precursor.

[b] $P<0.05$.

Bacterial lipid synthesis in leprosy tissues

Lepromatous tissue obtained from four different patients were studied for the incorporation of [^{14}C]-acetate in the tissue as well as in the bacteria present in the tissue (table 2). It is evident that both the tissue and bacteria incorporate [^{14}C]-acetate into lipids. Such incorporation was drastically reduced in the heat killed tissue. If the incorporation in total lipids is a relevant metabolic event, then one should be able to identify the various lipid components with the [^{14}C]-label. In table 3 the radioactivity associated with the various lipid components separated by thin layer chromatography are shown. It is clear from this, while incorporation is distributed in several lipid components, the pattern of incorporation is different in normal and infected skin. In infected skin there was much less incorporation into cholesterol as well as diglycerides. An unidentified spot of radioactivity was seen in

Table 2. Incorporation of [^{14}C]-acetate into total lipids of *M. leprae* and its host tissue.

			Tissue lipids from		Bacillary lipids from	
Biopsy number	Bacteriological Index[a]	Morphological Index[b]	Control tissue (cpm)	Heat killed tissue (cpm)	Control tissue (cpm)	Heat killed tissue (cpm)
325	5+	2%	47295	2405	17505	1300
335	6+	3%	49250	800	19440	780
402	6+	3%	12045	1000	15455	1030
408	6+	3%	11720	600	3550	260

[a] Bacteriological Index is calculated as nos of bacilli/field

1-10/100/fields	1+
1-10/10 fields	2+
1-10/1 field	3+
1-100/field	4+
1-1000/field	5+
1000/field clumps	6+

[b] Morphological Index is calculated as percentage of stained bacilli as solid forms: an indication of viable proportion of the total bacilli.

Table 3. Radioactive distribution of [^{14}C]-acetate in the neutral lipids of normal and *M. leprae* infected skin.

	cpm (%)	
Lipids	Normal skin	Infected skin
Cholesterol	12.3	2.3
Unknown	—	5
Triglycerides	36.3	20.5
C_{12}–C_{14}		
Palmitic	19	11.8
Stearic		
Cholesterol	8.8	34.5
Mono- and diglycerides	3	2.7
Glycolipids/phospholipids	20.3	23.5
	99.3	99.3

infected skin, which was not seen in normal skin. It will be shown later that this spot is probably derived from bacteria.

Effect of drugs on lipid synthesis by bacteria

Lepromatous tissue containing *M. leprae* was exposed to the precursor in presence and absence of the drug dapsone using eleven different infected tissues (table 4) and with rifampicin for five infected tissues (table 5). It was observed that the total

Table 4. Effect of dapsone on the incorporation of [^{14}C]-acetate into lipids of *M. leprae.*

	[^{14}C]-Acetate incorporation into total lipids^a of dapsone-sensitive *M. leprae*				[^{14}C]-Acetate incorporation into total lipids^a of dapsone-resistant *M. leprae*		
Biopsy No.	Control	Dapsone (10 μg/ml)	Bacteriological load^b	Biopsy No.	Control	Dapsone (10 μg/ml)	Bacteriological load
277	14	0	4+/2%	261	32	63	4+/4%
282	36	3	—	306	11	27	5+/1%
295a	47	25	5+/3–4%	325	35	39	5+/2%
298	25	20	6+/4%	340	63	72	—
299	50	37	6+/4%	382	39	64	6+/3%
302	12	5	2+/1%	397	100	100	6+/1%
315	29	13	3+/1%	408	54	70	6+/3%
317	40	31	6+/3%	Mean±SD	48±28^d	62±24^d	
332	96	68	5+/6%				
335	53	0	6+/3%				
402	87	74	6+/3%				
Mean±SD	44±27^c	25±28^c					

[a] Ratio of incorporation into bacteria to that into the tissue (%).

[b] $\frac{\text{Bacteriological Index}}{\text{Morphological Index}}$

[c] $P<0.05$.

[d] $P<0.001$.

Table 5. Rifampicin effect on the incorporation of [^{14}C]-acetate into lipids of *M. leprae.*

	[^{14}C]-Acetate incorporation^a into total lipids of *M. leprae*, sensitive to rifampicin				[^{14}C]-Acetate incorporation^a into total lipids of *M. leprae*, resistant to rifampicin		
Biopsy No.	Control	Rifampicin (5 μg/ml)	Bacteriological load^b	Biopsy No.	Control	Rifampicin (5 μg/ml)	Bacteriological load^b
295a	47	26	5+/3–4%	261	32	36	4+/4%
317	40	28	6+/3%	277	14	18	4+/2%
332	96	68	5+/6%	282	36	94	—
397	100	62	6+/1%	302	12	52	3+/1%
335	53	49	6+/3%	306	11	14	5+/1%
Mean±SD	67±28	47±19^c		325	35	41	5+/2%
				340	63	70	—
				382	39	66	6+/3%
				402	87	100	6+/3%
				408	54	56	6+/3%
				Mean±SD	38±24^d	55±29^d	

[a] Ratio of incorporation into bacteria to that into the tissue (%).

[b] $\frac{\text{Bacteriological Index}}{\text{Morphological Index}}$

[c] $P<0.05$

[d] $P<0.001$.

synthesis of lipids in the bacteria in the presence of dapsone or rifampicin was drastically reduced, in all these cases. The difference in the amount of incorporation between the control and drug treated tissue was statistically significant. If one were to look at lipid components form the bacteria separated from these tissues under the above conditions, it would be clear that the total reduction in lipids was also reflected in the reduced level of incorporation in the presence of dapsone. Virtually most components showed lower level of incorporation in the presence of dapsone and rifampicin.

However, if the lepromatous tissues are derived from patients suspected to harbour resistant bacteria to the drugs under administration or have been proved to have relapse, then the total incorporation into lipids of *M. leprae* is not reduced in the presence of the drug. The data in tables 4 and 5 provide the total incorporation of the label into lipids of the bacteria obtained from suspected drug resistant patients for dapsone and rifampicin respectively. It is clear that the drug has no effect on the corporation into lipids of bacteria indicative of the resistance. The levels of synthesis in the control and drug treated, are not statistically significant.

The control value from the 7 and the 11 samples in table 4 were comparable, but drug treated samples markedly differed showing sensitivity of the bacteria for dapsone in the 11 cases and resistance of the bacteria to the drug in the 7 cases studied. The level of incorporation of the precursor into lipid components was higher whenever drug resistant strains were tested as compared to incorporation in the absence of this drug. We have no explanation for this observation at present. But a clearer idea could be obtained when the drug resistant bacteria are studied in a cell-free system.

Correlation of DOPA method and acetate method

M. leprae from the tissues of the 8 patients were also tested for their sensitivity/resistance to the drugs rifampicin and dapsone by another method developed in our laboratory (Ambrose *et al.*, 1974; 1978) using labelled DOPA. Uptake of DOPA and its conversion to other metabolities is one of the characteristic feture of live *M. leprae* (Prabhakaran *et al.*, 1968; Prabhakaran, 1973). We observed that the drug reduced drastically the uptake of DOPA in the sensitive strains, while it had very little or no effect on DOPA uptake by the resistant strains. These results corroborate well with the drug sensitivity/resistance in the bacteria when the uptake of labelled acetate is measured in the presence of the drugs.

Bacteria from one of the tissues (FMR 299) were tested for sensitivity to dapsone by the mouse foot pad technique (Shepard, 1960). Harvests were carried out after 6, 8 and 10 months of growth in the mice. The results indicate that the bacteria from the patient were sensitive to dapsone as was indicated by the [^{14}C]-acetate uptake system.

Discussion

Experiments carried out with *M. lepraemurium*-infected liver and spleen tissue showed that acetate could be incorporated into mycolic acids but this was not the case with *M. leprae* obtained from skin of infected human subjects. Labeling in mycolic acids could not be obtained in the human bacilli probably for two reasons: a slow metabolic turnover of this lipid or the pathway for the biosynthesis of these acids involves a different precursor.

But we found that there was a significant incorporation in the chloroform-methanol extractable lipids in tissues containing *M. leprae* as well in the components separated after thin layer chromatography. Furthermore, the observation that dapsone and rifampicin decreased the percentage of label incorporated in the lipid portion of tissues containing *M. leprae* from patients resistant to the drug, while in resistant strains, the acetate incorporation into lipids was not inhibited correlated with the clinical status of the patient.

The absence of a significant difference in the incorporation of label in the control and drug treated normal skin indicated that the drug was probably effecting bacilli and not the metabolism of the tissue components. Biopsies which were examined for [^{14}C]-acetate incorporation into lipids, were also used to study [^{3}H]-DOPA uptake method to test drug sensitivity. The correlation between these two methods was quite good. Bacilli from one of the patients were inoculated into the mouse foot pad and the sensitivity to dapsone was shown even in the mouse.

Screening for drug sensitivity using [^{14}C]-acetate is rapid and results can be obtained within a period of 15 days. It is more informative than the method using [^{3}H]-DOPA uptake because acetate can be traced in the various lipid fractions, especially triglycerides and fatty acids. There is no ambiguity in the results and reproducibility appears to be good. The level of radioactive incorporation is fairly good unlike the incorporation of precursors into DNA (Nath *et al*, 1982). Bacilli with a Morphological Index of even 1% shows an uptake of the precursor.

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Changes in rat liver mitochondrial lipids in vitamin A deficiency

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Abstract. The alterations in the lipid profiles of rat liver mitochondria due to vitamin A deficiency were studied. The amount of total lipids and phospholipids were decreased with a concomitant increase in triglycerides and cholesterol levels in mitochondria, isolated from vitamin A-deficient animals. Of particular significance was the observation that the content of lysolecithin, a potent cytolytic agent, was increased. An analysis of individual fatty acids showed that the percentage of polyunsaturated fatty acids was decreased significantly in vitamin A deficiency. Further, mitochondria from vitamin A-deficient animals, when incubated in 0.1 M Tris-HCl buffer (pH 7.4) *in vitro*, produced increased amounts of malondialdehyde and lipofuchsin pigments indicating increased susceptibility of the mitochondrial membrane to peroxidative damage. These results suggest a possible role of vitamin A in the prevention of the decomposition of structural lipids.

Keywords. Vitamin A deficiency; rat liver; mitochondrial lipids; peroxidation.

Introduction

Specific membrane functions are known to be associated with the structural complexities and interactions of protein and lipid molecules (Singer and Nicholson, 1972). Permeability properties of cellular membranes can be markedly influenced by the fatty acid composition of the phospholipids, and in particular by their degree of unsaturation (Chapman, 1975; Chapman and Wallach, 1968). Similarly, the relative amounts of cholesterol and the regulation of acyl chain lengths are responsible for controlling membrane fluidity (van Deenen, 1965). The role of carotenoids in maintaining the fluidity of cell membrane has also been suggested (Rottem, 1980). Evidence has been presented to suggest that the mitochondrial electron transport chain of animal tissues is regulated by lipids (Jolliot, 1972).

The role of vitamin A in regulation of the structural and functional properties of biomembranes is well documented (Dingle and Lucy, 1965). Vitamin A forms an integral part of rat liver plasma membrane, endoplasmic reticulum and of erythrocyte ghosts (Mack *et al.*, 1972). Incorporation of orally administered [^{3}H]-retinol into rat liver plasma membrane has been demonstrated (Adhikari *et al.*, 1978). Antioxidant property of vitamin A has been suggested and it is shown to inhibit *in vitro* catalysis of linoleic acid peroxidation (Jayanthibai and Krishnamurthy, 1979). Several reports have described that lipid composition of subcellular membranes is

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Abbreviations used: TLC, Thin layer chromatography; GLC, gas-liquid chromatography; TBA, thiobarbuturic acid; PUFA, polyunsaturated fatty acids.

modified by dietary alterations, and affects the membrane-assoiated functions (Sun *et al.*, 1979). Robison *et al.* (1980) have shown that dietary retinol deficiency is directly responsible for the excessive accumulation of lipofuscins in retina and loss of photoreceptor cells. In the present investigation, we show that dietary vitamin A deficiency induces profound alterations in the lipid composition of rat liver mitochondria. These changes are reflected in membrane-linked processes. Quantitative changes in fatty acid composition result in peroxidative derangement and loss of succinic dehydrogenase activity.

Materials and methods

Chemicals

Phospholipids were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Cholesterol and fatty acid methyl esters were procured from British Drug House, Bombay and V.P. Chest Institute, New Delhi, respectively. Chromosorb P, coated with ethylene glycol succinate was obtained from Applied Science Laboratories, State College, Pennsylvania, USA. Sodium succinate was Koch-Light product. All other reagents and solvents were of analytical grade. Solvents were redistilled prior to use.

Animals

Weanling male rats of Wistar strain (body wt. 30–35 g), having low reserves of liver vitamin A, were obtained from mothers, fed on marginal levels of the vitamin. They were divided into two groups and maintained on a nutritionally adequate but vitamin A-deficient diet (Adhikari and Vakil, 1980). Control rats were administered orally 50 I.U. retinyl palmitate in 0.5 ml groundnut oil per day and pair-fed with deficient animals, which were given only oil. The animals were killed after about 5 weeks when the deficient rats lost weight for 2 to 3 consecutive days.

Isolation of mitochondria

Liver was quickly excised, chilled and homogenised in 0.25 M sucrose (pH 7.4) using a Potter-Elvehjem homogeniser with a teflon pestle. Mitochondrial pellet was isolated, following the method described by Weinback (1961). The relative yield of mitochondria in terms of total liver proteins was comparable (about 7.3%) in control and experimental groups.

Lipid analyses

Total lipids were extracted with chloroform: methanol (2 : 1, v/v) mixture (Folch *et al.*, 1957) and estimated colorimetrically (Bragden, 1951). Cholesterol and total phospholipid contents were determined colorimetrically from total lipid extracts by the methods of Sperry and Webb (1950) and Bartlett (1959), respectively. Triglycerides were separated by thin layer chromatography on silica gel G using petroleum ether : diethyl ether : acetic acid (82 : 18 : 1) and estimated colorimetrically (Schlierf and Wood, 1965). Separation of phospholipid components was performed by thin layer chromatography (TLC) as described by Daniels *et al.* (1966). Each phospholipid spot was identified by exposure to iodine vapour and quantitated by determining the phosphorous content (Bartlett, 1959). The recovery of the applied phospholipid was almost total.

Gas-liquid chromatography

Further, mitochondrial lipids were saponified with alcoholic KOH and extracted with petroleum ether : diethyl ether (1 : 1, v/v) mixture. The aqueous layer was acidified and methyl esters of the free fatty acids were prepared as described by Rao and Bandyopadhyay (1978). These were separated by gas-liquid chromatogrphy (GLC) using 20% ethylene glycol succinate on chromosorb P (60–80 mesh) packed into a stainless steel column (¼" o.d. × 6 ft). Column temperature was 195° C and nitrogen was used as the carrier gas. The peaks were identified using authentic esters of fatty acids and relative distribution of fatty acids was calculated by triangulation method (base × ½ height of the peak).

Measurement of peroxidative products

Isolated mitochondria were suspended in Tris-HCl buffer (pH 7.4) and incubated at 37° C in air for 4 h. Aliquots were withdrawn at regular intervals and mixed with 0.67% thiobarbituric acid (TBA) in 10% trichloroacetic acid. The mixture was heated in a boiling water bath for 15 min, cooled and centrifuged. Peroxides formed were measured in the supernatant after reaction with TBA reactants (at 535 nm) in a Bosch and Lomb spectrophotometer. Results were expressed in terms of nmol of malonaldehyde formed (Ambe and Tappel, 1961). Peroxidizable index was calculated by multiplying the number of double bonds with various factors as described by Bieri and Poukka (1970). Simultaneously, another aliquot of the mitochondrial suspension was withdrawn and extracted with chloroform : methanol (2 : 1, v/v). Fluorescence of lipofuchsins in the solvent phase, was measured in terms of their emission intensity having characteristic spectra with excitation maxima in the range of 360–380 nm and emission maxima at 430–460 nm in a Hitachi spectrofluorometer (Chio and Tappel, 1969). Quinine sulphate was used as a reference standard.

Diene conjugates

Blood was collected, oxalated and centrifuged at 600 *g* for 15 min. Lipids in plasma as well as in mitochondrial suspension were extracted with $CHCl_3$: CH_3OH (2 : 1, v/v) mixture. The solvent was evaporated to dryness under nitrogen and the residue was dissolved in heptane. Characteristic ultra-violet absorption spectra of the diene conjugates in lipid extracts were recorded from 220 to 280 nm in a Beckman DB spectrophotometer (DiLuzio, 1972).

Enzyme Assay

Succinic dehydrogenase activity was estimated spectrophotometrically using sodium succinate as the substrate and dichlorophenol-indophenol as endogenous electron acceptor (Caplan and Greenwalt, 1968). Protein was estimated using bovine serum albumin as standard (Miller, 1959).

Results

Lipid composition of mitochondria

Results on the effect of vitamin A deficiency on the lipid composition of mitochondria are summarised in tables 1 and 2. Total lipid and phospholipid levels

Table 1. Effect of vitamin A deficiency on lipid composition of rat liver mitochondria.

Lipid component	Control	Vitamin A-deficient
	(μg/mg protein)	
Total lipids	228.0±6.0	128.0±9.2†
Triglycerides	7.6±1.2	12.5±1.5*
Cholesterol	6.9±0.3	11.5±1.6*
Lipid phosphorus	6.2±0.8	3.9±0.6*
Cholesterol : phospholipid Molar ratio	0.10	0.25

Mitochondrial lipids were extracted and analysed as described in the text. Triglycerides were separated by thin-layer chromatography and estimated colorimetrically. Results are averages of 6 values ±S.E. *$P<0.05$; †$P<0.001$.

Table 2. Effect of vitamin A deficiency on individual phospholipids of rat liver mitochondria.

Phospholipid component	Control	Vitamin A-deficient
	(μg/mg protein)	
Cardiolipin	18.0±2.7	11.8±1.2
Phosphatidyl serine	ND**	ND**
Sphingomyelin	4.0±0.7	1.3±0.2
Phosphatidyl choline	73.8±7.6	32.8±2.7†
Phosphatidyl ethanolamine	55.0±6.4	39.0±4.3*
Lysolecithin	2.2±0.2	3.0±0.2*

Individual phospholipids were separated by TLC as described in the text. Each spot was scraped, eluted and digested with 10 N H_2SO_4 and perchloric acid. Phosphorous was estimated after digestion. Results are averages of six experiments ±S.E. *$P<0.05$; †$P<0.001$ **ND—Not detected.

(table 1) were significantly lower whereas cholesterol and neutral lipids (triglycerides), were higher in vitamin A-deficient group compared to that in the pair-fed control ones. Cholesterol: phospholipid molar ratio was 2.5-fold higher in the deficient group. The data on the relative concentration of individual phospholipids (table 2) revealed that the contents of phosphatidyl choline and phosphatidyl ethanolamine, two major phospholipids of the mitochondrial membrane, were decreased significantly in vitamin A deficiency. Sphingomyelin did not contribute appreciably to the mitochondrial lipids and phosphatidyl serine was not traced.

However, the level of cardiolipin, specific mitochondrial phospholipid, was decreased in vitamin A deficiency. It is interesting to note that lysolecithin concentration was increased in vitamin A-deficient mitochondria.

Fatty acid composition of mitochondria in vitamin A deficiency

The relative distribution of the fatty acids in liver mitochondria of vitamin A deficient and control rats is shown in table 3. Saturated fatty acids, namely, palmitic and stearic, were predominently present in the mitochondrial membrane and were increased to some extent in the deficient group. However, total polyunsaturated fatty acids (PUFA) ranged from 18 to 20% of total fatty acids in the deficient group, while the membrane from normal rat contained about 30 to 32% PUFA. Further, a significantly marked decrease in linoleic, palmitoleic and arachidonic acids was observed due to vitamin A deficiency, whereas no appreciable difference was noted in oleic acid level. Thus, about 70% decrease in the unsaturation of membrane-bound lipids was observed. Similarly, the peroxidisable index, which represents the peroxidisability of PUFA, was lower in vitamin A deficient group.

Table 3. Changes in mitochondrial fatty acid composition in vitamin A deficiency.

Fatty acid component	Control	Vitamin A-deficient
	(% of total fatty acids)	
$C_{14:0}$	13.2±1.6	13.6±2.0
$C_{16:0}$	23.5±3.1	28 0±2.9
$C_{16:1}$	3.5±0.5	2.1±0.3
$C_{18:0}$	13.2±1.8	23.1±3.2*
$C_{18:1}$	14.4±2.1	16.2±1.9
$C_{18:2}$	14.5±1.6	9.6±1.2*
$C_{18:3}$	Not traceable	Not traceable
$C_{20:4}$	16.9±2.0	8.3±1.2**
Total PUFA	31.3	18.1
Peroxidisable index	81	43

Fatty acids were extracted by diethyl ether from the saponified mitochondrial lipids, following acidification. Methyl esters of the fatty acids were prepared and separated by GLC as detailed in the text. Peroxidisable index was calculated from the number of double bands of fatty acids. Results are averages of six determinations ±S.E. *$P<0.05$; **$P<0.01$.

Peroxidative damage to mitochondria in vitamin A deficiency

Figure 1 shows the time-course of the formation of lipid peroxides, measured in terms of TBA-reactant malonaldehyde, during the exposure of mitochondria to atmospheric oxygen at 37° C. The concentration of malondialdehyde, the peroxidative product of structural lipids, was increased in the incubation mixture at a

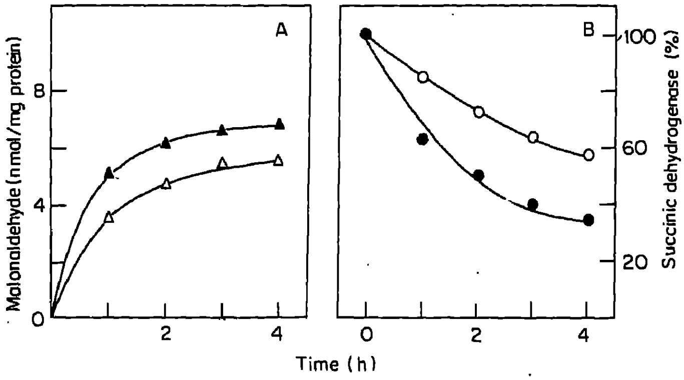

Figure 1. Measurement of TBA positive material reactants and succinic dehydrogenase activity in isolated mitochondria.

Isolated rat liver mitochondria were suspended in Tris buffer (pH 7.5) and incubated at 37° C in air. Aliquots were withdrawn at intervals and TBA positive material reactants (Δ), in terms of malonaldehyde (MA) formed, were measured. Succinic dehydrogenase activity (O) was determined spectrophotometrically using sodium succinate as the substrate. Initial values (0 min, taken as 100%) for the enzyme activity (Δ min/g re protein) were 0.63 and 0.41 for control (Δ, O) and vitamin A-deficient (▲, ●) groups, respectively.

much faster rate in vitamin A-deficient sampels. After 4 h of incubation, nearly 25% more preroxidative products were accumulated compared to that in the control mitochondria. The higher extent of damage to the mitochondrial membrane in vitamin A deficiency was further reflected in a significant decrease in succinic dehydrogenase activity during the course of lipid peroxidation. After 4 h of peroxidation, vitamin A deficient mitochondria retained only 35% of the initial (0 min) enzyme activity, compared to about 58% in vitamin A-fed animals.

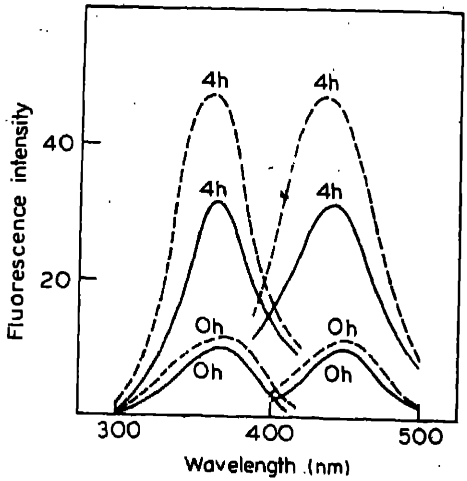

Figure 2. Development of fluorescence in isolated rat liver mitochondria.

Mitochondrial suspensions were incubated in air for 4 h and lipids were extracted. The mixture was centrifuged and fluorescence spectra in the solvent phase were recorded at different wavelengths. (——), Control; (-----), vitamin A-deficient.

Development of Schiff-base type fluorescent pigments, during *in vitro* incubation of mitochondria *in vitro* is plotted in figure 2. Initially, the quantities of the fluorescent pigments in both the groups were insignificant. With increasing time, however, the fluorescence intensity, which is directly proportional to the rate of formation of peroxidative products, increased rapidly in vitamin A-deficient mitochondria compared to that in mitochondria from the control group. After 4 h, nearly 1.5 times more fluorescent products were accumulated in the deficient mitochondria than that in control group. These represent the chromophoric molecular damge site and the extent of damage to biological membrane (Tappel, 1972).

Figure 3 shows the estimation of lipoperoxidation, measured by recording the absorption spectra of lipid extracts from 230 to 260 nm, characteristic of conjugated diene peroxides. It can be seen that mitochondrial total lipids, either from control or vitamin A-deficient rat liver, did not manifest the typical conjugated diene spectrum (figure 3A) indicating the absence of lipoperoxides *in vivo* in rat liver. However, the UV absorption spectra of plasma lipids from both the groups revealed the presence of diene conjugates having the typical maximum at 233 nm. About 25% increase in diene conjugation in vitamin A deficient group was observed compared to that in the control.

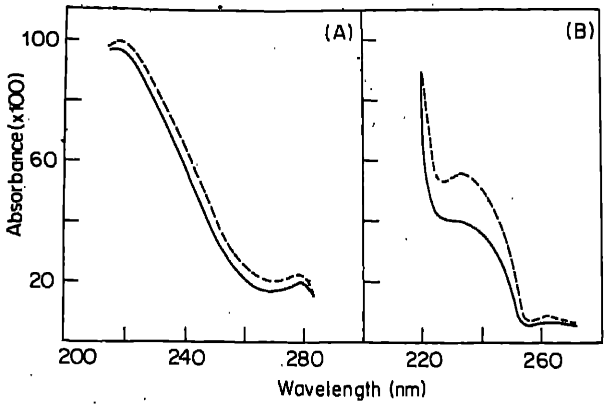

Figure 3. Diene conjugation absorption spectra of blood plasma and of mitochondrial lipids.

Blood plasma and liver mitochondrial lipids were extracted with $CHCl_3:CH_3OH$ (2:1, v/v) and taken in heptane. Absorption spectra were recorded at 220 to 280 nm. (——), Control; (- - - -), vitamin A-deficient.

Discussion

Mitochondrial system was selected for the present study, as it represents the endoplasmic reticulum structure and about 90% of the total lipids are in the membrane (Henning and Heidrich, 1974). Accumulation of neutral lipids (table 1) with concomitant reduction in phospholipids in vitamin A deficiency indicate that enzymic conversion of α,β-diglycerides and triglycerides to phospholipids as described by Weiss *et al.* (1958) is decreased. Similar changes in testicular lipid profiles in vitamin A-deficient rat have been reported (Gambal, 1966). Similarly, higher accumulation of cholesterol (table 1) may be attributed to the observed decrease in cholesterol side chain cleavage enzyme activity in vitamin A deficiency (Jayaram *et al.*, 1973). Further, cholesterol: phospholipid molar ratio, which governs the integrity and fluidity of biomembranes (Finkelstein and Cass, 1967), was increased in vitamin A deficiency (table 1). This observation is of some physiological relevance, since osmotic permeability coefficient in the membranes is also a function of this molar ratio. Significant reduction in phosphatidylcholine and phosphatidyl ethanolamine (table 2) in vitamin A deficiency may be due to an impairment of exchange reactions, since these are synthesized mainly in the endoplasmic reticulum, and then transferred to mitochondrial membranes (Moreau *et al.*, 1974). Similarly, vitamin A nutritional status of rat is shown (Chandra *et al.*, 1982) to affect liver mitochondrial phospholipase activity, which mediates the hydrolysis of phospholipids. Higher amount of lysolecithin may also accentuate the fragility of the mitochondrial membranes. Reduction in total number of double bonds of polyunsaturated fatty acids in vitamin A deficiency may be attributed to impaired metabolism of linoleic and arachidonic acids (table 3).

The functional implications of these changes in lipid composition were studied by measuring their peroxidation products. Significantly higher rate of formation of TBA reactants like malonaldehyde (figure 1), indicates the peroxidative changes in mitochondrial membrane in vitamin A deficiency. Inhibition of lipid peroxidation by dietary retinol has been reported (Kartha *et al.*, 1978). Similarly, addition of retinol reduces *in vitro* lipid peroxidation of fatty acids by rat tissue homogenates (Green *et al.*, 1964). We have estimated that the mitochondrial preparation from control rats contained about 0.2 μg retinol/mg protein; whereas it was not traced in vitamin A deficient samples. Thus retinol present in ioslated mitochondria, augments their antioxygenic potential and prevents lipid peroxidation (Kartha and Krishnamurthy, 1978). However, other peroxidation products such as diene conjugates, formed as a consequence of oxidation of phospholipids prior to malonaldehyde formation (DiLuzio, 1972), could not be traced in mitochondria but were found in higher concentration in vitamin A-deficient rat plasma (figure 3). These lipoperoxides are present *in vivo* only in the adipose tissues and not in the paranchymatous organs (Glavind, 1972). These extremely toxic hydroperoxides may be quickly degraded to malondialdehyde and other compounds or/are readily released into blood for enzymic detoxification in erythrocytes.

Further, the formation of lipofuchsins, the inert fluorescent pigments (Tappel, 1972), was also increased in vitamin A deficiency (figure 2). Robison *et al.* (1980) have shown that dietary vitamin A deficiency is directly responsible for the excessive accumulation of lipofuchsin in retina and loss of photoreceptor cells. Though, free radical scavenging property of vitamin A is not well established (Pryor, 1978), activities of free radical control enzymes such as catalase and glutathione peroxidase decrease in vitamin A deficiency (Peter and Bryden, 1979). This glutathione system is shown to prevent the formation of fatty acid peroxides (Koster *et al.*, 1982).

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Intrinsic multiple antibiotic resistance markers for competitive and effectiveness studies with various strains of mung bean rhizobia

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Abstract. Ten strains of *Rhizobium* sp. with multiple antibiotic resistance markers were used for competitive and efficiency studies with mung bean var. ML 5. All the strains showed significant increase in grain yield and so also for nitrogenase activity except MO 5. Nitrogenase activity correlated very well with grain yeild. The compatibility of strains varied from 17 to 50%. The intrinsic multiple antibiotic markers for strain identification were found to be stable after passing through soil and host conditions and could be used for ecological studies. It was further revealed that the overall efficiency of a strain is the combined effect of characters like compatability, competitiveness and inherent capacity to fix nitrogen.

Keywords. Intrinsic multiple antibiotic markers; competitiveness; ecological studies; effectiveness.

Introduction

Of the various nitrogen fixing systems, the symbiosis of *Rhizobium* with leguminous plants is the most effective system contributing annually at the rate of 35×10^6 tonnes to the nitrogen balance of the ecosystem (Burris, 1977). The major controlling factors contributing to the efficiency of inoculated strains are reported to be host genotype (Minchin *et al*, 1978; Mytton, 1978; Poi and Kabi, 1979) and ability of strain to dominate over the native rhizobia for nodulation (Herridge and Roughley, 1975; Gibson *et al*, 1976), in addition to its inherent capacity to fix nitrogen (Johnson and Beringer, 1975). Various valid methods such as serological technique (Dudmen and Brockwell, 1968), immunfluorescence microscopy (Bolhool and Schimdt, 1970), antibiotic resistance markers (Obaton, 1971) have been employed to understand the contribution of introduced rhizobia with respect to nodulation. Due to close correlation between results obtained using immunological and antibiotic resistance characters (Schwinghamer and Dudman, 1973) as well as the convenience of nodule sampling procedure, the intrinsic multiple antibiotic resistance markers were used to study the competitive and effectiveness of the introduced strains of *Rhizobium*.

Abbreviations used: Amp, Ampicillin; Ery, erythromycin; Str, streptomycin; Gen, gentamycin; Nal, nalidixic acid; Tet, tetracycline; Can, chloramphenicol; kan, kanamycin.

Materials and methods

Screening

Ten strains of *Rhizobium* sp. (cowpea group) used for competitive studies were screened for their intrinsic antibiotic resistance markers (table 1) using antibiotic disc assay method on Bergersen's medium (Bergersen, 1961). The discs (Span Diagnostics, Surat) used were streptomycin, 10; kanamycin, 30; nalidixic acid 30; chloramphenicol, 30; tetracycline, 30; gentamycin, 10; erythromycin, 15; and ampicillin, 10 μg/disc.

Experimental layout

Seeds of mung bean var. ML 5 were treated with charcoal based culture of 10-strains of *Rhizobium* sp., and sown in randomised block design (net plot area 5.4 sq.m.). Uninoculated seeds served as control. Phosphorus was applied at 40 kg P_2O_5/ha through single super phosphate as a basal dose.

Observations

At 45 days of growth: Five plants from each plot were uprooted and observations for the number of nodules, dry weight (wt) of nodules, plant height, dry wt of plants were recorded. Nitrogenase activity was measured by the acetylene reducing technique (Hardy *et al.*, 1968) by gas liquid chromatograph (Gas chromatograph, Nucon Model 5560) using a porapeak R column.

Competitiveness: The isolates of thirty nodules from five plants were screened for intrinsic antibiotic markers as explained already. The per cent competitiveness was calculated according to formula

$$\%\ \text{Competitiveness} = \underset{\text{in experiment}}{\%\ \text{marked nodules}} - \underset{\text{in control.}}{\%\ \text{marked nodules}}$$

At harvest: Crop was harvested at maturity and observations for total dry matter and grain yield were recorded.

Results

Intrinsic antibiotic spectra of Rhizobium strains

Out of ten strains used, strains M 10 and GMBS 1 were resistant to only one antibiotic; strains KM-1, MO^{-5} 5, PL 1, M 1 and Niftal (Tal 169) to two antibiotics; 32 HI to three antibiotics and strains M 73002 and M 73001 to four and five of the antibiotics tested (table 1). The frequency of resistance to different antibiotics was in the order of Amp>Ery=Str>Gen=Nal>Tet=Can>kan.

Nitrogen fixation of efficiency in mung bean under field conditions

The efficiency of ten inoculated strains in relation to number of nodules per plant, nodule dry wt per plant, plant height, dry wt per plant and nitrogenase activity was observed. The data are given in table 2. Out of these five parameters, nodule number, nodule weight and nitrogenase activity were found to be significantly higher with inoculation. The inoculation resulted in higher number of nodules with all the

Table 1. Antibiotic resistance spectra of different strains of *Rhizobium* sp. (cowpea group).

Strains	Antibiotic spectrum							
	Ery	Amp	Tet	Can	Gen	Str	Nal	Kan
M 10	*s*	*s*	*s*	*s*	*s*	*r*	*s*	*s*
GMBS 1	*s*	*s*	*s*	*s*	*s*	*r*	*s*	*s*
KM 1	*s*	*s*	*s*	*s*	*s*	*r*	*r*	*s*
Niftal (Tal 169)	*s*	*s*	*s*	*s*	*r*	*r*	*s*	*s*
MO 5	*s*	*s*	*s*	*r*	*s*	*s*	*r*	*s*
M 73001	*r*	*r*	*r*	*s*	*r*	*s*	*r*	*s*
PL 1	*s*	*r*	*s*	*s*	*s*	*s*	*s*	*r*
32 HI	*r*	*r*	*r*	*s*	*s*	*s*	*s*	*s*
M 73002	*r*	*r*	*s*	*r*	*r*	*s*	*s*	*s*
M 1	*r*	*r*	*s*	*s*	*s*	*s*	*s*	*s*

s – Sensitive; *r* - resistant.

Table 2. Effect of *Rhizobium* inoculation on ancillary characters of mung bean ML 5.

Treatments	No. of nodules/ plant	Nodule dry wt./ plant (mg)	Plant height (cm)	Dry weight/ plant (g)	Nitrogenase activity nM C_2H_4/h/g dry nodule
Uninoculated control	6.2	22.5	18.7	1.13	1310
M 10	8.7	28.6	22.2	1.20	2846
GMBS 1	9.2	24.5	21.0	1.60	3991
KM 1	4.0	15.0	18.4	1.42	4565
Niftal (Tal 169)	8.3	27.8	19.7	1.04	8526
MO 5	7.3	23.0	19.0	1.28	1672
M 73001	7.2	15.9	19.7	1.42	4799
PL1	7.9	17.6	18.5	1.23	2763
32 HI	10.1	28.8	20.4	1.23	5432
M 73002	7.4	19.4	17.9	1.29	4735
M 1	6.6	23.8	16.7	1.13	10002
S.Em. ±	1.01	3.40	1.07	0.18	860.7
C.D. at 5%	2.98	10.01	N.S.	N.S.	1042.52

strains except KM 1 which was, however, at par with control and M 1. Seven strains *viz.* M 1, M 73001, MO 5, M 73002, PL 1, Niftal (Tal 169) and M 10 were at par with control whereas strains GMBS 1 and 32 HI were significantly higher than control but at par between themselves. The per cent increase in nodule number in strains GMBS 1 and 32 HI was 48.3 and 62.9 respectively. In terms of nodule dry weight, M 10, Niftal (Tal 169) and 32 HI gave significantly higher nodule dry weight over KM 1, M 73001 and PL 1. Other strains, however, were at par amongst themselves. A significant increase in nitrogenase activity with all the strains over uninoculated control except strain MO 5 which showed non-significant increase of 27.6% was observed. Strains PL 1 and M 10 were superior over MO 5 whereas strain GMBS 1, KM 1, M 73002 and M 73001 were at par among themselves but significantly higher than M 10. Strain M 1 showed highest activity followed by Niftal (Tal 169) and 32 HI. Strain M 1 and Niftal (Tal 169) showed significantly higher activities over all other treatments.

The effect of inoculation on final dry matter and grain yield is given in table 3. The final dry matter was significantly higher with four strains *viz.* GMBS 1, PL 1, KM 1 and M 1 with a percentage increase of 28.4, 28.9, 36.4 and 43.8 respectively, whereas strains Niftal (Tal 169), M 73001, and 32 HI with per cent increase of 7.5, 7.5 and 9.5 were at par with control. However, strains M 73002, MO 5, M 10 showed a decrease in final dry matter. All the test strains resulted in significantly higher grain yield with percentage increase ranging from 15–60%. Strain M 1 gave highest grain yield (875 kg/ha) followed by Niftal (Tal 169) (823 kg/ha).

Table 3. Performance of various strains of *Rhizobium* sp. on yield of mung bean ML 5.

Treatments	Final dry matter (kg/ha)	Per cent increase/ decrease over control	Grain yield (kg/ha)	Per cent increase/ decrease over control
Uninoculated control	5886	–	532	–
M 10	5277	−10.3	752	41.3
GMBS 1	7561	+28.4	806	51.5
KM 1	7718	+36.4	758	42.5
Niftal (Tal 169)	6330	+ 7.5	823	54.7
MO 5	5127	−12.6	612	15.0
M 73001	6330	+ 7.5	803	32.1
PL 1	7589	+28.9	684	28.3
32 HI	6441	+ 9.4	662	24.4
M 73002	4685	−20.4	647	22.6
M 1	8273	43.6	851	80.0
S.Em. ±	861.69	–	18.84	–
C.D. at 5%	1042.52	–	54.98	–

Correlation coefficient and regression between yield and yield attributing characters

The correlation coefficient values and regression equations between yield and yield attributing characters are given in table 4. The grain yield was found to be the best correlated with dry matter having r value of 0.58 with a regression of $\hat{Y} = 401.57 + 0.0479\,X$ followed by nitrogenase activity and the nodule weight with r value of 0.278 and 0.185 respectively, whereas, the nodule number was found to be poorly correlated ($r = 0.059$). Similarly, a poor correlation of 0.033 was observed in case of nodule number *vs* nitrogenase activity, but on the other hand nodule weight *vs* nitrogenase activity showed a correlation of 0.25. A good correlation ($r = 0.4$) was observed between nitrogenase activity and dry matter with a regression of $Y = 3704.46 + 0.196\,X$.

Table 4. Correlation coefficients and regression equations for yield and yield attributing characters in mung bean.

Characteristics	Correlation coefficient (r)	Regression equation
Dry matter *vs.* grain yield	0.58	$\hat{Y} = 401.70 + 0.0479\,X$
Nodule No./plant *vs.* grain yield	0.059	$\hat{Y} = 696.4 + 2.04\,X$
Nitrogenase activity *vs.* grain yeild	0.278	$\hat{Y} = 707.2 + 0.76$
Nodule wt./plant *vs.* grain yield	0.185	$\hat{Y} = 630.36 + 3.63\,X$
Nodule No./plant *vs.* nitrogenase activity	0.033	$\hat{Y} = 4363.6 + 32.31\,X$
Nodule wt./plant *vs.* nitrogenase activity	0.25	$Y = 4335.02 + 111.69\,X$
Nitrogenase ability *vs.* dry matter activity	0.40	$\hat{Y} = 3704.46 + 0.196\,X$

Table 5. Competitive ability of introduced strains of *Rhizobium* sp. with native rhizobia (mung bean ML 5).

Rhizobium sp. (Strains)	Competitiveness (per cent)
M 10	20.0
GMBS 1	50.0
KM 1	33.3
Niftal (Tal 169)	50.0
MO 5	16.6
M 73001	40.0
PL 1	16.6
32 HI	20.0
M 73002	28.5
M 1	40.0

Competitive ability

The competitive ability of introduced strains over native population in forming nodules is given in table 5 which ranged from 17 to 50.0%. The highest competitiveness was found with GMBS 1 and Niftal (Tal 169) (50.0%) followed by M 73001 and M 1 (40% each), whereas strains MO 5 and PL 1 could compete only to the extent of 17%.

The antibiotic spectra of isolates obtained during competition studies showed that a large number of native rhizobia were sensitive to all the test antibiotics (53%). It was further observed that frequency of resistance to single and double antibiotics was higher than multiple resistance (figure 1).

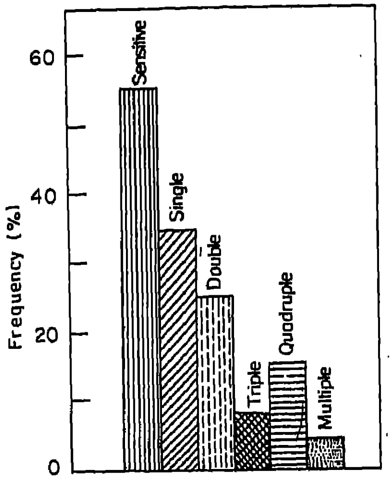

Figure 1. Frequency of multiple antibiotic resistance in cultures of *Rhizobium* sp. isolated from root nodules of mung bean.

Discussion

Screening for multiple drug resistance and also for antibiotic resistance revealed, that most of the strains were resistant to ampicillin and streptomycin, and least to chloramphenicol, tetracycline and kanamycin. Similarly reports on occurrence of multiple drug resistance and higher resistance to antibiotics like penicillin, streptomycin, erythromycin and low frequency to tetracycline and chloroamphenicol have been reported by Cole and Elkan (1979) and Kahlon (1980). Frequency distribution of isolates made from root nodules of mung bean (figure 1) during competitive studies revealed that with the increase in resistance to number of antibiotic markers their frequency decreased. In an extensive screening of soil bacterial isolates, Partiskaya and Corelova (1976) and Cole and Elkan (1979) also demonstrated low frequency of multiple drug resistance. This is in confirmation with the theory of probability, according to which chances of an incidence decreases with the increase in limitations to which it is subjected. Similarly, the susceptibility of majority of isolates to antibiotics may be due to the fact that these bacteria have not been exposed to antibiotics in the natural habitat.

The low degree of increase in dry matter production in some of the inoculated strains could possibly be on account of comparatively low efficiency of these strains in terms of nitrogen fixation, thereby overall less contribution to the pool of source but the extent of transfer towards the sink remaining the same. The low efficiency of these strains is evident from the low values of nitrogenase activities (2848 and 1672 units for M 10 and MO 5 respectively) as compared to other strains. In case of strains M 73002, although the activity was reasonably good but its nodule weight was found to be less as compared to uninoculated control. Similarly, low values were also observed in few other strains like KM 1, M 73001 and PL 1 which might be due to small size and fewer number of nodules. The distribution pattern of root nodule on root system might be governing the utilization of biologically fixed nitrogen by the host. The beneficial effects of *Rhizobium* inoculation towards increasing the nitrogenase activity and yield have also been observed by various workers (Subba Rao, 1976; Sinha, 1978; Minchin *et al.*, 1978).

The correlation coefficient between yield and yield attributing characters indicated that yield is correlated to dry matter ($r = 0.58$) which in turn is dependent on nitrogenase activity ($r = 0.4$) hence the nitrogenase activity would be most reliable parameter for estimating the yield. Moreover, nitrogenase activity can be assessed after a short period of crop growth and the possibility of handling large number of samples at a time makes it a more feasible parameter for assessing the efficiency of inoculation strain and in computing the yield.

Results reveal that higher yield of the crop is dependent not only on the inherent nitrogen fixing capacity (nodulation on nitrogenase activity) on the strains but also on their competitive ability. For instance strains M 1 and Niftal (Tal 169) show high degree of nitrogenase activity as well as competitive ability (40-50%) and consequently highest grain yield were also obtained due to these strains. High yield obtained through strains GMBS 1 is also attributed to its *v.* high competitive ability and moderate nitrogenase activity. On the other hand strain 32 HI showed moderate nitrogenase activity but extremely poor competitive ability and consequently resulted in poor grain yeild. As an extreme case, the two strains PL 1 and MO 5 showed very low nitrogenase activity as well as competitive ability and these two strains produced the lowest grain yield. Thus it is seen that presence of both the qualities, *viz.*, high nitrogenase activity as well as high competitive ability is essential for obtaining optimum symbiotic association leading to high yield of the crop. Consideration of competitive ability (or dominance against native rhizobia) especially, in case of highly promiscuous legume such as groundnut (*Arachis hypogea*) and other cowpea group of legumes, while screening for the most efficient rhizobial strains was also advocated by Gaur *et al.* (1974). The reisolation of multiple antibiotic resistance marked strains from the root of nodules of mung bean after passing through the soil environment and host indicate that these markers are stable and could be used successfully for such ecological studies.

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The effect of propranolol on rat brain catecholamine biosynthesis*

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Abstract. The effect of propranolol on the levels of catecholamine in different parts of rat brain has been studied. The catecholamine contents of different regions were lowered by the drug. Dopamine β-hydroxylase activity was also reduced, both *in vivo* and *in vitro*. Propranolol is taken up by the brain tissue and the uptake is time-dependent. These results suggests that reduction in brain catecholamine levels and dopamine β-hydroxylase activity may be one of the possible ways through which the drug manifests its clinical effects.

Keywords. Catecholamine biosynthesis; propranolol; β-blocker; dopamine β-hydroxylase.

Introduction

Propranolol, one of the most popular β-adrenergic blocking agents, has been used effectively in certain clinical cases of hypertension (Buhler *et al.*, 1972; Bravo *et al.*, 1975), cardiac arrhythmias and angina pectoris (Livesley *et al.*, 1973). It easily crosses the blood brain barrier and is concentrated in the central nervous system (CNS) (Masuoka and Hansson, 1967). It is a lipid soluble compound and high concentration of this compound accumulates in the brain of rabbits (Black *et al.*, 1965), rats (Masuoka and Hansson, 1967) and monkeys (Hayes and Cooper, 1971). Catecholamines have been implicated in the pathophysiology of hypertension (de Champlain *et al.*, 1967; Chalmers and Wurtman, 1971; Doba and Reis, 1974). In hypertensive patients, an increased plasma dopamine β-hydroxylase activity has been reported by several investigators (Schanberg *et al.*, 1974; Nagatsu *et al.*, 1976; Lamprecht, 1979). Biochemical effects of propranolol on catecholamine levels and biosynthetic enzyme activities are not well understood. Hence, it was of interest to study the effect of this drug on dopamine β-hydroxylase activity and catecholamine levels in the rat brain.

Materials and methods

Adult male rats of Charles Foster strain (100-150 *g*) were obtained from Central Drug Research Institute animal colony. They were maintained on a standard animal house diet. dl-Propranolol-HCl was purchased from Sigma Chemical Company, St. Louis, Missouri, USA. For *in vivo* studies rats were divided into 4 groups of 50 rats each and treated as follows:

Abbreviations used: CNS, Central nervous system; E, epinephrine; NE, norepinephrine; DA, dopamine.

*C.D.R.I. Communication No. 3053.

Group I:	Rats administered normal saline served as controls.
Group II:	Rats received dl-propranolol-HCl (20 mg/kg body wt.) intraperitoneally in normal saline for 3 days at 24 h intervals.
Group III:	Rats were treated as in group II for 5 days.
Group IV:	Rats were treated as in group II for 7 days.

The rats were killed by cervical dislocation, 1 h after the last dose. The brains were quickly removed, kept on ice and the brain-stem, hypothalamus, and corpus striatum were dissected.

Catecholamine estimation

The levels of epinephrine (E), norepinephrine (NE) and dopamine (DA) of different parts of the brain were estimated as reported earlier according to the method of Malherbe (1971) with certain modifications. The oxidation of catecholamines was carried out by iodine solution as described by Sadavongvivad (1970).

Dopamine β-hydroxylase assay

The enzyme activity was assayed as described earlier (Srivastava and Kapoor, 1979, 1980) by a modification on the procedure of Nagatsu and Udenfriend (1972) using tyramine as substrate. The reaction product octopamine was cleaved into *p*-hydroxybenzaldehyde and its absorbance was read measured at 330 nm using a Beckman spectrophotometer. *In vitro* effect of propranolol on the enzyme activity in brain extracts (prepared in 0.32 M sucrose; 0.5 M phosphate buffer, pH 6.2 added in the reaction mixture) was studied using different concentrations of the drug. Protein was estimated according to Lowry *et al.* (1951).

Propranolol estimation

Tissue propranolol levels were estimated by a slightly modified procedure of Shand *et al.* (1970). Brain homogenate (16% wt/v) prepared in 0.1 N HCl was made alkaline by the addition 1 ml of 1 N NaOH (1 to 4 ml homogenate and extracted into 12 ml heptane containing 1.5% isoamylalcohol. After centrifugaion 10 ml of the organic phase was extracted into 1.5 ml of 0.1 N HCl and the fluorescence of the acid phase was measured in an Aminco-Bowman spectrophotofluorometer (excitation maximum 295 nm; emission maximum 360 nm, uncorrected).

Results

As shown in table 1 a progressive decrease in the levels of E, NE and DA in the brain stem was seen. While the E and NE levels remained almost unchanged even after 5 days of treatment with dl-propranolol. Dopamine β-hydroxylase activity was also inhibited progressively showing 23% inhibition at 7 days. In hypothalamus, E and DA decreased progressively, (table 2) upto 5 days, while NE level declined gradually at all periods of treatment. Dopamine β-hydroxylase activity was maximally inhibited (29%) at 7 days. NE and DA contents of corpus striatum were altered similarly as in the brain-stem while the levels of E was not significantly affected (table 3). Dopamine β-hydroxylase activity showed marginal inhibition (19%) at 7 days. The *in vitro* treatment, addition of propranolol inhibited the enzyme maximally by (32%) at 1 mM (table 4). The decrease in catecholamine

Table 1. Effect of dl-propranolol treatment on catecholamine level and dopamine β-hydroxylase activity of rat brain-stem.

Treatment	Catecholamine (ng/g wet tissue)			Dapamine* β-hydroxylase activity (units)
	E	NE	DA	
Control (3)	178± 8.5	274±26.4	240±23.8	1.3±0.11
3 days (3)	144±10.9	242±25.3	210±13.6	1.1±0.08
5 days (3)	133±12.5	235±22.7	166±20.5	1.0±0.09
7 days (3)	138±13.2	230±32.6	117±15.8	1.0±0.05

*nmol octopamine formed per mg protein per min.

All values are mean ±S.E.M. of 6 observations.

Numbers in parenthesis indicate the number of brain-stems pooled.$P<0.01$.

Table 2. Effect of dl-propranolol treatment on catecholamine level and dopamine β-hydroxylase activity of rat hypothalamus.

Treatment	Catecholamine (ng/g wet tissue)			Dopamine* β-hydroxylase activity (units)
	E	NE	DA	
Control (4)	546±11.2	647±63.5	264±25.9	1.4±0.13
3 days (4)	471±30.6	583±58.4	248±23.7	1.3±0.11
5 days (4)	438±29.3	560±63.0	209±21.6	1.1±0.11
7 days (4)	426±43.3	530± 5.5	204±39.8	1.0±0.08

*nmol octopamine formed per mg protein per min.

All values are mean ±S.E.M. of 5 observations.

Number of hypothalamus pooled is indicated in parenthesis. $P>0.01$.

levels in various regions of the brain may be correlated with the decreased activity of the enzyme upon treatment with propranolol. Propranolol was also taken up by the brain. The drug levels were 0.93, 1.70 and 2.58 μg/g wet tissue at 3, 5 and 7 days of treatment, respectively.

Discussion

In the present investigations, it is observed that propranolol administration results in progressive decline in the levels of catecholamines in different parts of the brain *viz.* brain-stem, hypothalamus and corpus striatum. Similarly, dopamine β-hydroxylase activity was also decreased in these parts of the brain. In the brain-stem, levels of E were decreased maximally at 5 days of propranolol administration. A continued decrease in the level of NE was observed in the three parts of the brain till the 7th day after the drug administration. Although the decrease in the

Table 3. Effect of dl-propranolol treatment on catecholamine level and dopamine β-hydroxylase activity of rat corpus striatum.

Treatment	Catecholamine (ng/g wet tissue)			Dopamine* β-hydroxylase activity (units)
	E	NE	DA	
Control (3)	130±12.8	178±16.9	336±22.5	1.1±0.09
3 days (3)	120±10.3	152±13.6	284±19.3	1.0±0.07
5 days (3)	119±10.7	147±15.1	266±21.6	0.9±0.05
7 days (3)	118±10.9	134±13.8	225±18.6	0.9±0.06

*nmol octopamine formed per mg protein per min.

All values are mean ±S.E.M. of 6 observations.

Number of corpus-striatum pooled is indicated in parenthesis. $P < 0.01$.

Table 4. *In vitro* effect of dl-propranolol on rat brain dopamine β-hydroxylase activity.

Propranolol (Mm)	Dopamine β-hydroxylase activity (units)
0	1.3±0.11
0.01	1.1±0.10
0.1	1.0±0.08
1.0	0.8±0.08

nmol octopamine formed per mg protein per min.

Values are mean ±S.E.M. of 6 observations. $P > 0.001$.

synthesis of NE could be due to the decreased activity of dopamine β-hydroxylase, that other enzymes like monoamine oxidase and catecholomethyl transferase have a role in the regulation of catecholamine levels cannot be ruled out. The decrease in DA level could also be due to the involvement of tyrosine hydroxylase. Bhagat (1979) has explained the relationship between decreased adrenal tyrosome hydroxylase activity and lowering of blood pressure. It was proposed that a decrease in this enzyme activity resulted in lowered NE synthesis leading eventually to a decrease in the amount of amine available for release.

A considerable amount of information has accumulated in the recent years to suggest that β-adrenoceptor blocking action of propranolol leads to hypotension and other therapeutic actions (Kelliher and Buckley, 1970; Srivastava *et al.*, 1973; Sharma *et al.*, 1979; Dollery *et al.*, 1973; Planz and Planz, 1981; Virtanen *et al.*, 1982; Bianchetti *et al.*, 1982), however, some investigators have presented evidence to suggest that these actions are independent of the β-adrenergic blocking activity of the compound (Leszkovszky and Tardos, 1965; Murmann *et al.*, 1966; Grana and Sossi, 1967). Several antihypertensive drugs have been reported to exert actions on noradrenergic pathways in the CNS and this is the possible site of action for propranolol as this compound accumulates to high levels in the CNS due to its ability to easily cross blood-brain barrier. Smits *et al.* (1980) have also reported that propranolol produces hypotension by an action within the CNS.

In our studies, we have shown that the levels of propranolol increase with the duration of the treatment indicating the availability of the drug in the brain. Myers *et al.* (1975) have also reported significant concentrations of propranolol in the brain of experimental animals and patients after prolonged oral administration. The progressive increase in the brain levels of propranolol may be due to the inhibitory effect it exerts on its own metabolism (Schneck and Pritchard, 1981) in the rat liver.

The present study reveals that the propranolol causes decline of catecholamine levels in brain parts indicating that biogenic amine homeostasis may be regulated by dopamine β-hydroxylase whose activity is modulated by the drug.

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Ureolytic and nitrifying bacterial heterotrophs from acid soils

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Abstract. Nitrification following ureolysis in soil samples from tea growing soils (pH 4.5–5.5) was found to be chiefly due to the activity of heterotrophic bacteria belonging to genera *Bacillus*, *Arthrobacter*, *Sporosarcina*, *Micrococcus*, *Clostridium*, *Pseudomonas* and *Proteus*. A correlation between the intensity of ureolytic activity of organisms in a given soil sample and the yield levels of tea was observed. In culture media the increase in the quantity of NH_4^+-N indicating ureolysis was not accompanied by proportional increase in biomass. Ureolysis and nitrification in sterile soil sample inoculated with the isolates improved through amendment of organic carbon to the soil.

Keywords. Acid soils; ureolysis; nitrification.

Introduction

While estimating microbial response to urea degradation in soil samples from tea plantations (pH 4.5–5.5), it was found that good ureolytic activity, detectable within 24 h of incubation with urea was generally from sections having a high yield of tea. The detection of nitrite—and nitrate-N in the samples following ureolysis suggested, that active nitrification was also in progress. Since pH optima for autotrophic nitrification is said to be efficient at a pH values above 7 (Boon and Laudelout, 1962; Engel and Alexander, 1960; Hofman and Lees, 1952; Loveless and Painter, 1968) and the ammonia release, has been reported to be toxic to *Nitrosomonas* and *Nitrobacter* sp. (Cremer and Fox, 1980), it was considered interesting to study nitrification following ureolysis in these soils. This is particularly significant in view of the findings of Ishaque and Cornfield (1976) who reported the absence autotrophic nitrifiers in acid tea soils. While the low population counts of autotrophic nitrifiers in these soils supported this view, inhibition to ureolysis by thiourea, (which resulted in reduced nitrification) raised doubts on the absence of autotrophic nitrification in these soils. In order to clarify these points, organisms responsible for the ureolytic and subsequent nitrification activity were, isolated from tea soils and characterised.

Materials and methods

Soil samples and yield of tea in these sections were obtained through the courtesy of the managers of the tea plantations located around Jorhat (26°44′ latitudes and 94° 10′longitude). Samples were collected at a depth of 15 cm from 6 sampling sites within each section (4–10 ha), mixed well and sieved after air drying. Eight sections of each type (good, medium, poor and virgin sections) were obtained from 3 different plantations (400–1000 ha). Samples from virgin sections were obtained from bamboo scurff forest which were situated within the plantation enclosures and not exposed to any form of cultivation.

To determine ureolytic and nitrification activity of the soils and of individual isolates, 5 g air dried soil samples were incubated with 2.5 ml of sterile water containing 60 mg of urea, or 72 mg of thiourea or 132 mg of ammonium sulphate. For determining nitrification activity of the isolates in the presence of thiourea, it was added in combination with urea and glucose. To study the influence of organic carbon amendment upon the ureolytic and nitrification activity of the isolates in sterile soil samples, glucose (1 g) or paraquat (0.2 ml of gramoxone) were introduced into the 5 g soil samples before sterilization. Paraquat was chosen to represent the organic carbon source through pesticides because gramoxone was the most popular herbicide used in the plantations under study. To determine extracellular urease activity, soil samples were pre-treated with 2.5 ml toluene for 30 min prior to incubation with urea (Bhavanandan and Sunderalingam, 1971). Urea was introduced in all cases at the time of incubation after pre-sterilization by exposure to ultra-violet (M) light. To compare nitrification activity of the soil samples in the presence of ammonium sulphate and urea and in combination with thiourea, 500 ml in 0.1 M solutions of ammonium sulphate, ammonium sulphate+thiourea, urea and urea+thiourea were eluted through 200 g soil columns previously dried with air. The eluate collected after 6 h were analysed for ammonium, nitrite and nitrate content. To estimate the improvement of ureolytic and nitrification activity of the samples previously showing poor activity, soil samples from the same source were divided into two sets, one set containing mixed culture of the isolates and the other without the inoculum and stored for two months. To prevent contamination of the samples from outside agencies, the mouth of the flasks were fitted with sterile cotton plungs. All the flasks from both the sets were left on the laboratory shelf under natural temperature fluctuations (25–30° C). At the end of this period, sterile urea was introduced into both sets of flasks and the ureolytic and nitrification activity after 24 h of incubation with urea was determined.

Quantative estimation of ureolysis and nitrification in all cases were made by extracting the incubated soil samples with 50 ml of 2 N KCl and 10 ml aliquots of the KCl extract were used for the determination of ammonium, nitrite and nitrate (Bremner, 1965). Appropriate blanks were always run.

Most probable number estimations were made by following Alexander (1965a) method 100–3 using *Nitrosomonas* and *Nitrobacter* enrichment media of Alexander and Clarke (1965).

Isolation of ureolytic strains were made both in Blake's (1946) media and in media containing 1 g glucose, 1 g NaCl, 0.8 g $MgSO_4$, 0.2 g KH_2PO_4 and 1 g $Na_2S_2O_3$ in 1 L water. Urea (6 g/L) sterilized as before, was introduced into the media at the time of inoculation. The same media was also used as base media for measuring starch hydrolysis [with $(NH_4)_2SO_4$ 13.2 g/L instead of urea for H_2S production (by introducing 0.02 g/L $FeSO_4$), for facultative autotrophism (with 1 g/L $NaNO_2$ or $(NH_4)_2SO_4$ 1 g/L in the absence of glucose] and for studying the influence of higher concentration of glucose (10 g/L) upon growth.

Test media and procedure to test starch hydrolysis, catalase activity, acetylmethyl carbinol formation, methyl red reaction, ammonia from peptone, reaction to lactose and glucose, growth in citrate, reaction in milk, motility test, stain procedures were all according to the Mannual of Microbiological Methods (Conn *et al.*, 1957a, b; Weiss, 1957). Observation on the colour of the colony were made both in peptone agar media (Clarke, 1965) prepared without indicator and in starch media.

Ammonia, nitrite and nitrate formation in culture was qualitatively detected using Nessler's reagent (Rand *et al.*, 1976), Griess-Ilosvay's reagent (Alexander, 1965b) and chromotrophic acid reagent (Rand *et al.*, 1976) respectively. For quantitative estimation of the compounds in culture media, inocula were introduced into test tubes with premeasured quantities of culture media and the entire inoculum with the culture were taken directly into the Kjeldahl distillation units at the end of the indicated period of incubation. In the case of time–course studies aliquots were withdrawn under asceptic conditions every 3 h or 24 h, centrifuged, separated from the biomass and then taken into Kjeldahl distillation units for determination of ammonium-, nitrite- and nitrate-N as according to the method of Bremner (1965). Protein estimation of the biomass was made with Folin reagent (Lowry *et al.*, 1961).

To detect whether the isolates produce ammonia in nitrogen-free media, the *Azotobacter* enrichment media of Clarke (1965) was used. Ammonia in the cultures was detected using Nessler's reagent.

Results and discussion

Intracellular ureolytic-nitrification activity in soil samples measured through released ammonium, nitrite and nitrate (Bremner, 1965) following 24 h of incubation with urea was markedly more in samples from sections having a high yield of tea. Similarly the ratio of ammonium-N: nitrate -N and ammonium-N: nitrite-N in good medium, poor and virgin sections which was 10 : 2 and 10 : 0.67; 10 : 0.38 and 10 : 0.8, 10 : 0.35 and 10 : 2.8 and 10 : 0.12 and 10 : 1.12 respectively, also suggested that in good yielding sections nitrification to nitrate was more rapid (table 1).

Since these differences in the ureolytic activity between the samples from the different types of sections disappeared when the samples were pretreated with

Table 1. Intracellular ureolytic-nitrification activity in soil.

Yield of tea	Ammonium-N (mg)		Nitrite-N (mg)		Nitrate-N (mg)	
	A	B	A	B	A	B
2540-2555 Kg (Good)	0.165-0.175	6-7.5	0.04-0.05	0.4-0.43	0.05	1.2-1.4
1028-1037 Kg (Medium)	0.18-0.19	2-3	0.04	0.06-0.07	0.05	0.2-0.22
511-517 (Poor)	0.2-0.23	0.39-0.49	0.5-0.56	0.14-0.16	0.06	0.015-0.017
Virgin	0.17-0.175	1.3-1.4	0.02	0.2-0.21	0.07	0.017

A – Pretreated with 2.5 ml toluene.
B – Not treated with toluene.

5 g air dired samples were used.

toluene, it can be infered that while the physical property of the samples were more or less the same for all sections, the rate of ureolysis was dependent upon viable organisms present in the soil at the time of urea amendment. However, as is evident from the high values for nitrate obtained in samples from poor yielding sections treated with toluene, inhibition to intracellular nitrification cannot be assessed in toluene treated samples. It is most likely that some chemical present in poor yielding sections reacts with toluene to give high values of nitrate-N.

In the presence of thiourea (table 2) ammonium was more readily extractable from the soil in samples amended with ammonium sulphate than in samples amended with urea. While this indicates inhibition to ureolysis by thiourea, the reduction in nitrification could not have been a consequence of reduced ureolysis.

Table 2. Effect of various nitrogen compounds on soil nitrification

Compound	Ammonium nitrogen (mg)		Nitrite nitrogen (mg)		Nitrate nitrogen (mg)	
	Poor soil	Good soil	Poor soil	Good soil	Poor soil	Good soil
Ammonium sulphate	67.53	71.97	10.0	13.0	4.7	5.5
Urea	40.5	37.0	5.0	7.6	3.7	6.4
Ammonium sulphate + thiourea	165.0	174.9	3.2	5.6	1.5	1.9
Urea + thiourea	25.5	20.5	0.5	1.0	0.7	1.5

500 ml of 0.1 M solutions of various compounds either singly or in combination were perfused through soil columns (200 g) in 6 h.

Because thiourea inhibited nitrification even in ammonium sulphate amended samples. As thiourea is a known inhibitor of autotrophic nitrifiers (Painter, 1970) existence of autotrophic nitrifiers in these soils is suggested. However, since NH_3 release has been reported to be toxic to *Nitrosomonas* and *Nitrobacter* (Cremer and Fox, 1980) rapid (24 h) nitrification in the presence of NH_3, as in samples incubated with urea, could only have been due to organisms, other than the known autotrophic nitrifiers. In contrast to this detection, nitrite and nitrate in soil dilution inoculated into *Nitrosomonas* and *Nitrobacter* media was possible only after 14 days of incubation. Furthermore, population density studies in either *Nitrosomonas* or *Nitrobacter* media showed that these types of organisms were rare in these soils. *Nitrobacter* could be detected only in November (35×10^3) in poor yielding sections, in August–November (75×10^5 to 45×10^3) in high yielding sections. *Nitrosomonas* could be detected almost throughout the year except between April to July in high yielding sections. The highest number recorded was in August (29×10^6). In poor yielding sections, they could not be detected between March to September and the highest number recorded was 10×10^5 in January. Thus, these findings suggest strongly that nitrification in these soils, was chiefly due to the activity of other nitrifiers. In support of this, Ishaque and Cornfield (1976) also reported the activity of heterortrophic nitrifiers in acid soils.

Organisms responsible for ureolytic activity recognized in solid media (method 3) through colour change of phenol-red incorporated into media, exhibited ureolysis 24–48 h after streaking. None of the ureolytic strains were able to grow in a completely mineral media, in the absence of organic carbon. Several strains were able to produce ammonia in nitrogen free medium. The general characters of the different isolates are described in table 3.

Ureolytic and nitrifying activity of the isolated strains evaluated through quantitative estimation of ammonium-, nitrite- and nitrate-N accumulated in the media of 10 days old cultures revealed, that nitrification following ureolysis occurred in several strains. The differences in the quantities of ammonium-, nitrite- or nitrate-N observed in these cultures indicated that ability to form nitrite or nitrate depended upon the strain. Although the detection of nitrate in most of the cultures after the 10 days incubation, suggested that auto-oxidation of nitrite could have taken place, the high quantity of nitrite detected in cultures of *Bacillus* spp. (No. 2), *Clostridium* spp. (No. 8) and *Micrococcus* spp. (No. 18), showed that nitrate formation in the culture may not entirely be due to auto-oxidation. Furthermore, as nitrite-N was recovered in some of the strains even after 10 days of incubation, the ability of the organisms to produce either nitrite, or nitrate is clearly a property of the strains (table 4).

Ureolytic and nitrifying activity of *Sporosarcina ureae* (No. 11), *Sporosarcina* spp. (Nos. 1, 22, 32 and 34), *Micrococcus* spp. (No. 18), *Proteus vulgaris* (No. 19) and mixture of *Bacillus pasteurii* (No. 5), *Bacillus freundenreichii* (No. 6), *Bacillus* sp. (No. 2) and *Arthrobacter* sp. (No. 3) in liquid media (method 3), estimated by withdrawing 10 ml aliquots under aseptic conditions revealed that ammonium-N in the media was detectable 12 h after inoculation, nitrite-N after 21–24 h and nitrate-N after 36–40 h.

Contrary to the expectation, that increase in ammonium-N as a result of ureolysis, was a function of the growth of the organisms with time, time-bound studies revealed, that ureolysis prevented proliferation of the organisms. The continued increase in the quantity of ammonium-N, unaccompanied by the increase in biomass, suggested that biochemicals needed for the proliferation of the organisms were not being synthesised during ureolytic activity. The influence of pH which rose sharply with the onset of ureolysis could also have contributed to growth retardation. Since the isolates grew well in a media containing $(NH_4)_2SO_4$ (1 g/L), of glucose instead of 1 g/L suggests that in glucose enriched media, ureolysis does not commence until glucose depletion. The increase in biomass, apparently ceases when ammonium-N becomes detectable in the media (figure 1).

Thiourea inhibited ureolysis in most of the strains. In sterile soil samples containing a combination of urea, thiourea and glucose inoculated with *Arthrobacter* spp. (Nos. 7 and 21), *Bacillus freudenreichii* (No. 6), *Bacillus* spp. (Nos. 28 and 36), *Sporosarcina* (Nos. 22, 34 and 35), *Micrococcus* sp. (Nos. 17 and 18), and *Proteus vulgaris* (No. 19), the quantity of ammonium-N detected 150–250 mg and nitrite-N + nitrate-N was 400–600 mg to 60 mg of urea after 24 h of incubation. Ureolysis without nitrification in presence of thiourea occurred in samples inoculated with *Sporosarcina* spp. (Nos. 1 and 24), *Bacillus pasteurii* (No. 5), *Bacillus* sp. (No. 13), *Clostridium* sp. (No. 8), *Arthrobacter* sp. (Nos. 3, 10 and 14) in samples inoculated with *Clostridium butyricum* (No. 27), *Sporosarcina* sp. (No. 35), *Bacillus* sp. (No. 2), *Micrococcus* sp. (No. 17) and *Proteus vulgaris* (No. 19), only nitrate-N could be recovered in the KCl extract.

Table 3. Distinguishing characters of isolates.

Isolate No.	Specific identification	Gelatin liquification	Growth in citrate	Amm. prodn. in nitrogen free media[a]	Acetyl methyl carbinol formation	Starch hydrolysis	Indole forming ability	Colour of colony in peptone agar	Remarks
1	2	3	4	5	6	7	8	9	10
	1. Gram positive or variable rods with heat resistant spores.								
	1.1. With catalase								
	1.1:1. H_2S from thiosulphate								
	1.1.1.1. Methyl red negative, reaction in glucose in milk neutral								
Bacillus									
9	*polymyxa*	+	+	+	–	–	+	White	
25	*lentus*	+	–	+	+	+	+	Purple	
6	*freudenreichii*	–	–	+	–	–	+	Cream white	
36		(+)	–	+	+	–	(+)	Cream white	
2		(+)	+	–	+	+	(+)	Cream white	
33		–	+	+	–	+	+	White	
31		–	–	–	+	+	+	White	
	1,1.1.2. Mythyl red positive, reaction in milk and glucose acidic.								
5	*pasteurii*	++	–	+	–	–	–	Cream	
	1.1.2. NOH_2S from thiosulphate, reaction in milk neutral, in glucose acidic.								
28		++	–	–	+	–	+	White	Methyl red –
4		+	+	–	–	–	+	Purple	Methyl red +
26		(+)	+	–	+	–	++	Dirty white	Methyl red –
13		–	+	+	+	–	+	Dirty white	Methyl red –

Table 3. *Contd.*

1.2. Without catalase

Clostridium									
27	*butyricum*	–	–	–	–	+	+	Dirty white	Methyl red +
8		–	–	–	–	–	+	Dirty white	Methyl red –

2. Gram positive rods older cultures coccoid. No heat resistant bodies catalase positive and H_2S from thiosulphate, reaction in glucose neutral, methyl red negative.

Arthrobacter									
10		+	+	+	+	–	+	Dirty white	Milk reaction acidic
7		–	+	+	+	+	+	Yellow	Milk reaction neutral
14		+	+	+	+	–	+	Purple	Milk reaction acidic
37		–	+	–	+	–	+	Black	Milk reaction neutral methyl red-d
21		++	+	+	+	–	+	Black	Milk reaction neutral methyl red-d
3		–	+	–	+	–	–	Dirty white	Milk reaction neutral
29		++	–	–	+	–	+	Purple	Milk reaction acidic

3. Gram negative rods without heat resistant bodies with catalase. H_2S from thiosulphate methyl red positive, milk reaction neutral, acid from glucose

Pseudomonas									
20		+	–	+	+	–	+	Yellow	
23		–	+	+	–	–	+	Yellow	
Proteus									
19	*vulgaris*	–	+	–	–	–	+	White	Acid from glucose, methyl red–d

4. Gram positive cocci, with heat resistant bodies, with catalase, reaction to glucose neutral.
4.1. H_2S from thiosulphate

Sporosarcina									
11	*ureae*	++	–	+	–	–	++	Dirty white	Acid from milk
16		–	+	+	–	–	+	White	Neutral in milk
1		++	–	–	–	–	+	Black	Neutral in milk

Table 3. *Contd.*

	4.2 No H_2S from thiosulphate milk reaction and glucose neutral.							
32	(+)	+	+	+	+	+	White	Methyl red +
34	+	−	−	+	+	(+)	White	Methyl red −
22	+	+	+	−	−	−	Cream	Methyl red −
24	−	+	+	−	+	+	Cream	Methyl red −
35	−	+	+	+	+	+	Cream white	Methyl red − c.
	5. Gram positive cocci without heat resistant bodies, milk reaction neutral.							
	5.1 With H_2S from thiosulphate							
Micrococcus								
17	++	+	+	−	+	++	Dirty white	Methyl red +, milk slowly turning acid
15	+	+	+	+	−	+	Purple	Methyl red −
12	++	+	+	+	−	−	White	Methyl red −
	5.2. Without H_2S from thiosulphate							
18	++	−	−	+	−	+	Dirty white	Methyl red −
30	+	−	−	−	−	(+)	Purple	Methyl red − d.

— = Negative; (+) = weak; + = strong; ++ = very strong; c = control; d = doubtful.

[a] The nitrogen-free medium was that described by Clarke (1965).

Table 4. Influence of amended carbon upon ureolytic and nitrifying property of the isolates.

Isolate No.	Ammonium-N (mg)				Nitrite-N (mg)				Nitrate-N (mg)			
	48 h incubation			10 day culture	48 h incubation			10 day culture	48 h incubation			10 day culture
	A	B	C	D	A	B	C	D	A	B	C	D
1	2	3	4	5	6	7	8	9	10	11	12	13
Bacillus												
5	640	4580	520	9800	210	260	2040	420	0	700	290	0
9	0	290	350	5270	35	0	100	280	87	35	100	0
29	170	380	380	5700	120	87	570	220	0	0	370	70
6	0	380	370	7500	87	260	930	280	0	0	490	1120
2	0	210	150	750	70	35	1080	750	30	0	100	140
36	0	290	0	6600	35	87	100	0	87	0	87	220
33	170	290	1480	240	35	0	67	0	87	350	92	230
31	0	350	1480	2460	120	17	1920	0	0	30	87	500
28	52	380	2100	210	310	120	1200	0	0	0	350	700
4	0	150	0	840	120	35	0	0	0	52	35	0
26	170	380	520	6880	120	50	960	0	0	0	260	560
13	0	470	70	1960	120	0	920	0	0	0	17	0
Clostridium												
27	470	640	260	580	280	170	0	0	0	0	470	140
8	0	210	87	1120	120	0	50	2100	0	0	37	500
Arthrobacter												
10	350	640	570	7800	560	120	50	0	0	0	17	860
7	0	210	610	810	120	80	150	0	0	0	80	280
14	2450	4060	170	5880	910	430	680	200	80	0	80	0

Table 4. *Contd.*

	37	97	122	260	140	126	0	1000	0	80	260	430	140
	21	0	170	0	350	470	0	0	0	80	120	0	140
	3	0	210	0	5230	35	0	280	80	80	80	80	0
	29	520	7700	170	560	120	1400	0	0	0	0	610	0
Pseudomonas													
	20	80	560	700	2240	120	80	30	0	80	0	30	700
	23	0	470	120	1110	30	80	30	0	80	0	20	42
Proteus													
	19	4810	5810	1220	7560	3270	170	2800	0	0	1920	30	1750
Sporosarcina													
	11	2180	6330	170	8960	900	1920	380	0	0	120	30	980
	16	430	380	0	7560	297	80	380	0	0	0	0	160
	1	170	470	140	1730	1100	80	610	0	0	0	170	0
	32	80	290	170	1640	120	0	520	0	80	260	170	520
	34	175	560	52	350	380	260	70	0	0	0	30	350
	22	0	380	120	2940	70	80	89	84	0	0	50	140
	24	0	470	1260	2260	0	0	0	0	200	80	30	490
	35	260	560	120	2450	1170	170	120	0	0	120	50	0
Micrococcus													
	15	170	260	520	7750	190	0	70	0	0	470	170	220
	17	3230	4580	210	3220	240	1570	610	0	0	0	1380	98
	12	3500	8430	1570	7000	1600	1130	590	470	0	170	350	0
	18	260	470	780	3150	120	80	1660	1960	0	0	610	120
	30	80	290	100	1820	120	80	0	0	0	0	600	350

A = Sterile soil as in nature 1% organic carbon.
B = Same as A, amended with 1 g glucose.
C = Same as A, amended with 0.2 ml gramaxone.
D = Culture media after 10 d incubation.

The isolates were inoculated into 5 g sterile soil samples and ammonium, nitrite- and nitrate-N accumulation in 10 day old culture medium.

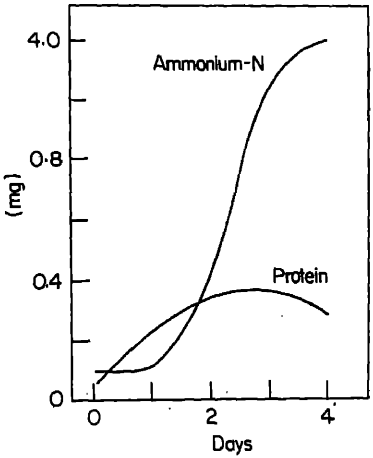

Figure 1. Ureolytic activity compared to growth measured in terms of protein, mg of protein and NH_4-N in 1 L culture media containing 10 g/L glucose.

Enhanced nitrification observed following addition of either paraquat (as gramaxone) or glucose, suggested that paraquat was being utilized as a source of carbon by the isolated species. It is also clear that organic carbon associated in nitrification (table 4). It is here significant that the strains such as *Bacillus* sp. (Nos. 6, 13, 26, 28, 31, 33 and 36). *Clostridium* sp. (No. 18), *Arthrobacter* sp. (Nos. 7 and 21), *Pseudomonas* sp. (No. 20), *Proteus vulgaris* (No. 19), *Sporosarcina* sp. (Nos. 1, 22, 24, 34 and 35) and *Micrococcus* sp. (Nos. 17 and 18) which yielded more ammonium in the presence of paraquat than in the presence of glucose were also the strains, which showed nitrification activity in the presence of thiourea.

Mixed cultures of isolates introduced into soil samples previously known to show poor ureolytic and nitrification activity, were able to establish themselves in the samples, thereby improving the ureolytic and nitrification property of the samples. The viability of the introduced organisms in the soil samples was established by comparing the ureolytic and nitrification activity of the controls with the

Table 5. Yield of ammonium, nitrate and nitrite nitrogen.

Soil types	NH_4-N (mg)	NO_2-N (mg)	NO_3-N (mg)
A	1.12	0	0.66
A+	5.75	1.0	0.54
B	4.74	0.5	0.82
B+	10.15	0.54	1.22

A — Poor activity type samples.

B — Good activity type samples.

A+ and B+ — The respective soil types with inoculum.
[illegible] soil samples stored in the laboratory for 2 months and amended with [illegible] mg urea in 2.5 ml sterile water 24 h before estimation.

inoculated samples, after being stored in flasks for a period of two months. While the inoculated samples showed a significant increase in both ureolysis and nitrification, the uninoculated samples showed only a slight increase thereby confirming that organisms were able to re-establish themselves in the samples (table 5).

Therefore, it can be concluded that in the soil samples studied, nitrification through heterotrophic strains was more predominant than autotrophic nitrifiers. Since the activity of these strains was decidedly more in sections having a good yeild of tea, it is logical to surmise that soil conditions in the sections concerned were congenial to growth and activity of these strains. However, since the reason for the reduction in the activity in poor yielding sections has not yet been cleared, it is difficult to estalish whether the condition of the cultivated species is dependent upon the strains in question or vice versa.

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Sugar-hydrolysing enzymes in gustatory mutants of *Drosophila*

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Abstract. The distribution of the isozymes of α-glucosidase in different parts of *Drosophila melanogaster* was examined. Two of the eight glucosidase bands are associated with tarsi. The gustatory mutations in the genes *gust*B and *gust*C greatly reduce tarsal glucosidase activity, but the enzyme deficiency is not correlated with inability to taste sugars.

Keywords. Neurogenetics; glucosidase; taste receptors; gustatory mutants.

Introduction

Dipterans possess a number of sugar hydrolysing enzymes. The α-glucosidases (EC. 3.2.1.20) cleave the 1,4α-glucosidic linkage, β-glucosidase (EC. 3.2.1.21) attacks β-glucosidic disaccharides and trisaccharides, and trehalase (EC. 3.2.1.28) cleaves the α-1,1 glucose-glucose bond. The glucosidases act on a broad-range of substrates although trehalase is relatively specific. Dethier (1955) found that glucosidase activity was present in the tarsal sensilla of blowflies. Hansen (1969) proposed that α-glucosidases act as receptor proteins for sugars. Although Hansen's working hypothesis has been entertained by several investigators, the arguments in its favour are mainly circumstantial (see review by Hansen, 1978). There is indeed little direct evidence to illuminate the exact physiological role of glucosidase in chemosensory reception.

We have obtained *gustatory* mutants in four different X-linked genes of *Drosophila melanogaster*, unable to taste sugars or salts (Rodrigues and Siddiqi, 1978, 1981). Some of these mutations specifically alter the electrophysiological responses of the taste hairs, which prompted us to examine the glucosidases of the gustatory mutants. In this paper we describe the tissue distribution of glucosidase activity in normal and mutant flies. In two of the *gust* mutants the sugar-hydrolysing activity, specific to tarsal hairs, is greatly reduced but glucosidase deficiency is not necessarily correlated with inability to taste sugars.

Materials and methods

Strain

The wild type strain was Canton Special (CS). The *gust* mutants are its derivatives (Siddiqi and Rodrigues, 1978). Flies were cultured on standard cornmeal medium.

Abbreviations used: CS, Canton Special; DTT, dithiothreitol; PMSF, phenyl methyl sulphonyl fluoride.

Enzyme preparation

Two to five days old flies were left in dry bottles to clean themselves of adherin food, then transferred to vials and frozen in liquid nitrogen. The heads and leg were separated by vigourous shaking followed by sieving through 0.8 mm nylo mesh. Legs, antennae and heads were spread on blotting paper; legs and antenna stick to the paper while the heads can be easily rolled off. Ten g of flies yield 1 g o heads and 50 mg of legs.

The legs (20 mg), heads (50 mg) and whole flies (100 mg) were separatel homogenised in 1 ml of 0.05 M potassium phosphate buffer (pH 7.2) containin 0.1 M KCl, 0.4 mM dithiothreitol (DTT), 1 mM EDTA and 0.5 mM pheny methyl sulphonyl fluoride (PMSF) and a mixture of protease inhibitors Homogenisation was carried out on ice, with 100 up-down strokes in a motor driven Potter-Elvehjem homogeniser. The homogenate was stirred for 2 h at 4°C and centrifuged for 1 h at 27,000 *g*. The supernatant was used as a source o enzyme.

Soybean trypsin inhibitor (1 mg/ml), pepstatin (1 μg/ml) leupeptin (1 μg/ml), antipain hydrochloride (1 μg/ml), benzamidine HCl (1 μg/ml), and EGTA (0.1 mM) were added to the homogenisation buffer to inhibit protease activity.

Non-denaturing gels

Enzyme extracts were electrophoresed in 7.5% acrylamide gel with 0.2% bisacrylamide. The resolving gel was polymerised in 0.03 M imidazole-HCl buffer (pH 7.2). The stacking gel (3.8% acrylamide with 0.1% bisacrylamide) was polymerised in 0.03 M imidazole HCl (pH 6.2). The cathode buffer contained 0.016 glycylglycine-imidazole (pH 6.7) and the anode buffer 0.03 M imidazole-HCl (pH 7.2). Electrophoresis was carried out at a constant current of 20 mA.

The enzyme was assayed as described by Tanimura *et al.* (1979). The gels were incubated in 0.5 M sucrose (or other sugars as required) in 100 mM sodium citrate NaH_2PO_4 (pH 5.5) for 30 min at 37° C and washed in distilled water, then soaked in 0.1% triphenyl tetrazolium chloride in 0.5 N NaOH at 30°C for 5 min. The stained gels were dried between two sheets of cellophane paper and scanned in an analytrol densitometer

Chemicals

Acrylamide, bisacrylamide, phenyl methyl sulphonyl fluoride (PMSF), soybean trypsin inhibitor, leupeptin hemi sulphate, antipain hydrochloride, benzamidine hydrochloride and EDTA were from Sigma Chemical Co., St. Louis, Missouri, USA. Sucrose and triphenyl tetrazolium chloride were from British Drug House, England.

Results

The polyacrylamide gel profiles of the glucosidases of *Drosophila melanogaster* were examined by Tanimura *et al.* (1979). They found 7 isozyme bands; 3 of the bands which were prominent were designated I, II and III. Extracts from entire flies, in our hands yield 8 bands of α-glucosidase activity whose (table 1) positions

Table 1. α-Glucosidase activity in limb-specific bands in normal and mutant flies.

	Activity (%) in D and E*
Canton Special (C.S.)	40
*gust*A	48
*gust*B	5
*gust*C	6
gust D	33

* Expressed as fraction of total activity in the gel.

are comparable with Tanimura's bands except that band E is not present in his gels. We have designated these bands A-H as shown in figure 1. Bands A, B and F in our gels correspond with Tanimura's I, II and III. When the gels are stained with raffinose, only one band appears near the top of the gel; this is β-fructofuranosidase (Huber and Mathison, 1975). Staining with trehalose gives a single rapidly

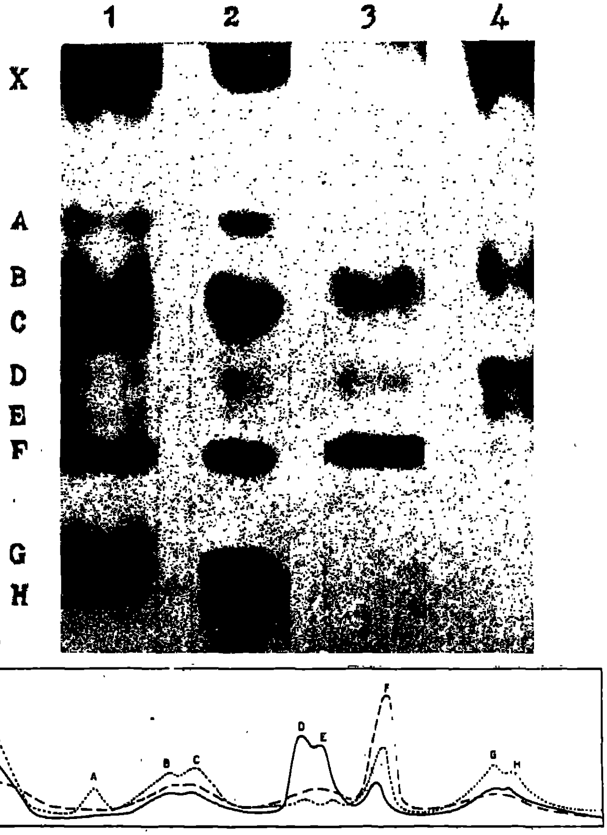

Figure 1. Electrophoretic species of glucosidase in normal *Drosophila* and its parts: 1. whole fly, 2. abdomen and thorax, 3. head and 4. leg. The band X near the top is a β-fructofuranosidase and strains with raffinose; A-H are α-glucosidases.

Densitometric tracing shows the profile of glucosidase isozymes in normal *Drosophila*: whole fly (.......); head (- - - -); legs (——).

migrating band of activity. Trehalose is not hydrolysed by α-glucosidase (Marzluf, 1969).

In order to examine the tissue distribution of the isozymes, extracts from separate parts of the fly, head, body and legs were electrophoresed. Figure 1 shows the glucosidase profile of different parts. The relative amount of activity in each band was estimated from densitometric scan of stained gels (figure 1). These are semi-quantitative estimates prone to errors arising from non-linearity of staining specially when the bands are too faint or too dense. Our estimates are averages of several gels. The standard error of these estimates ranges from 20 to 30%.

The band A is present in the body but absent from head and leg. The molecular weight of this band, 250,000 against 200,000 reported by Tanimura *et al.* (1979). The bands D and E are greatly enriched in the legs; the two bands together account for about 40% of the activity in the limbs D and E thus, seems to be the glucosidases associated with the tarsal sensilla. The molecular weight of these enzymes is 120,000 daltons. About 12% of the activity in the legs is contributed by a 75,000 dalton species designated as F; this is the predominant band in the head and is most likely the same as band III of Tanimura.

Glucosidase in gustatory mutants

Extracts from legs and heads of four different gustatory mutants *gust*A, *gust*B, *gust*C and *gust*D were examined. The glucosidase profiles are shown in figure 2. The mutants *gust*A and *gust*D are very similar to wild type but *gust*B and *gust*C show greatly reduced enzyme activity in the limb specific bands D and E. The proportion of total glucosidase activity in bands D and E in the four mutants, estimated by densitometric scanning of stained gels is given in table 1. In *gust*B, a prominent rapidly migrating band of activity as well as a band close to C appears in extracts of leg and head (figure 3).

In order to decide whether the enzyme deficiency in the mutant strains is specifically related to the mutation in question, we isogenised the *gust* stocks by repeated back-crossing to wild type, followed by selection for the *gust* phenotype for 3 generations. A number of lines from these crosses, segregating for *gust*B and *gust*C, were then examined for limb-specific glucosidases. The glucosidase deficiency was inseparable from the gust mutation (table 2).

Only one allele of *gust*C was available but two independent alleles of *gust*B showed similar enzyme deficiency.

Discussion

The taste sensilla of *Drosophila* are present on its proboscis and tarsi. The neural organisation of the labellar and the tarsal hairs is similar. A typical gustatory hair contains five sensory neurons four of which are chemosensory. One of these neurons called *S* responds to sugars while two others(L_1 and L_2) respond to salts. The gustatory mutations studied by us affect the labellar and the tarsal responses alike as judged by the proboscis extension test and electrophysiology (Rodrigues and Siddiqi, 1978). We have examined mainly the glucosidase of tarsal hairs.

The isozymes, D and E appear to be associated with the tarsal sensilla. This assumption is reinforced by the fact that two of the gustatory mutants are deficient in these two bands. The enzyme deficiency of the mutants, however, does not pro-

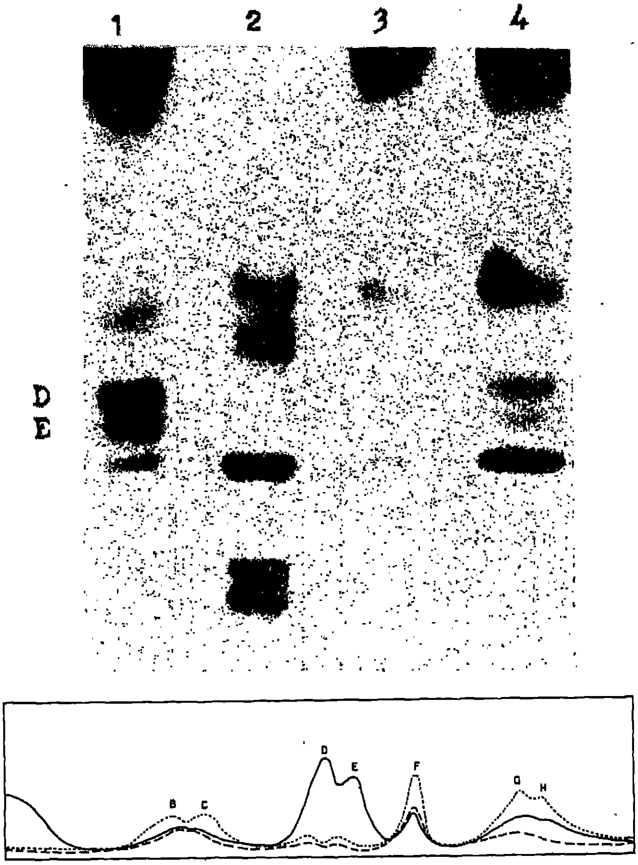

Figure 2. The pattern of glucosidase activity in the tarsi of gustatory mutants: 1. wild type, 2. *gust*B, 3. *gust*C and 4. *gust*A. Bands D and E are greatly reduced in *gust*B and *gust*C but *gust*B has increased activity at unusual positions.

Tracings of glucosidase profile of gustatory mutants: Leg extracts of *gust*A (____); *gust*B (.......) and *gust*C (------).

Table 2. α-Glucosidase activity in recombinant progeny from crosses segregating for *gust*B and *gust*C.

Recombinants			Lines examined	Activity in D and E bands
1. *y*	*cv*	*gust*B	5	Absent
y	*cv*	+	3	Present
*gust*B	*v*	*f*	2	Absent
+	*v*	*f*	2	Present
2. *y*	*cv*	*gust*C	3	Absent
y	*cv*	+	2	Present
*gust*C	*v*	*f*	2	Absent
+	*v*	*f*	1	Present

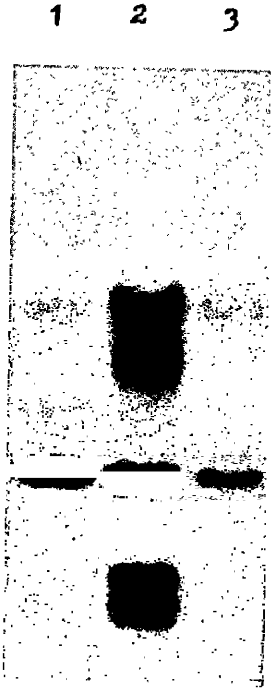

Figure 3. Glucosidase activity from head of normal and mutant flies: 1. wild type (CS), 2. *gust*B and 3. *gust*C. Note the presence of abnormal bands in *gust*B.

vide any clue to the functional involvement of glucosidase in chemoreception. The mutant *gust*A which is specifically blocked in behavioural and electrophysiological responses to pyranose sugars has a normal glucosidase profile; *gust*B and *gust*C which lack the limb-specific glucosidase, on the other hand, show apparently normal sugar responses; these mutants respond abnormally to salts. The simplest predictions of Hansen's hypothesis , that glucosidase itself acts as the receptor for sugars, are therefore, not borne out.

The fact that two different salt-insensitive mutations affect glucosidase levels in the sensilla is somewhat of an enigma. It is possible that the genes *gust*B and *gust*C are not directly concerned with glucosidase structure; rather an alteration in the normal organisation of the tarsal sensilla affects the accumulation or stability of the enzyme. Recent electrophysiological and behavioural experiments in our laboratory show that *gust*B responds to salts in an unusual way. Instead of being repellants NaCl and KCl act as attractants, at the same time eliciting greatly enhanced firing from sensory neurons (Rodrigues, Arora and Siddiqi, unpublished results). An interesting possibility is that *gust*B causes the appearance of salt acceptor sites on the *S* neuron, normally responsive to sugars alone. At any rate there is evidence of peripheral changes in the sensory receptors in this mutant. The same cannot be said of *gust*C, which has apparently normal firing responses to sugars and salts.

Lastly, although our results fail to provide any support for Hansen's hypothesis, they do not, strictly speaking, exclude the involvement of glucosidase in sensory reception. Some glucosidase activity appears in unusual position in the gels of *gust*B. The mutants, although deficient in the bands D and E, do not altogether lack glucosidase activity in their sensilla.

Acknowledgement

We thank Dr. K. S. Krishnan for many suggestions and helpful discussion.

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Purification and kinetic mechanism of 5,10-methylenetetrahydrofolate reductase from sheep liver

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Abstract. 5,10-Methylenetetrahydrofolate reductase (EC 1.1.1.68) was purified from the cytosolic fraction of sheep liver by $(NH_4)_2SO_4$ fractionation, acid precipitation, DEAE-Sephacel chromatography and Blue Sepharose affinity chromatography. The homogeneity of the enzyme was established by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, ultracentrifugation and Ouchterlony immunodiffusion test. The enzyme was a dimer of molecular weight 1,66,000 ± 5,000 with a subunit molecular weight of 87,000 ± 5,000. The enzyme showed hyperbolic saturation pattern with 5-methyltetrahydrofolate. $K_{0.5}$ values for 5-methyltetrahydrofolate menadione and NADPH were determined to be 132 μM, 2.45 μM and 16 μM. The parallel set of lines in the Lineweaver-Burk plot, when either NADPH or menadione was varied at different fixed concentrations of the other substrate; non-competitive inhibition, when NADPH was varied at different fixed concentrations of NADP; competitive inhibition, when menadione was varied at different fixed concentrations of NADP and the absence of inhibition by NADP at saturating concentration of menadione, clearly established that the kinetic mechanism of the reaction catalyzed by this enzyme was ping-pong.

Keywords. 5,10-Methylenetetrahydrofolate reductase; purification; ping-pong mechanism.

Introduction

5,10-Methylenetetrahydrofolate reductase (EC 1.1.1.68), a flavoprotein, catalyses the conversion of 5,10-methylenetetrahydrofolate ($5,10\text{-}CH_2\text{-}H_4$folate) to 5-methyltetrahydrofolate ($5\text{-}CH_3\text{-}H_4$folate), the major storage form of folic acid in bacteria, animals, and plants (Blakley, 1969). It is also the transport form in mammalian systems. The reaction catalysed by this enzyme is shown in equation (1).

$$5,10\text{-}CH_2\text{-}H_4\text{folate} + \text{NADPH} + H^+ \rightleftharpoons 5\text{-}CH_3\text{-}H_4\text{folate} + \text{NADP}^+ \quad (1)$$

In addition, $5\text{-}CH_3\text{-}H_4$ folate transfers its methyl group to homocysteine to form methionine which is a precursor of S-adenosyl-methionine (SAM). SAM transfers its methyl groups to a variety of acceptors. The reaction catalysed by the reductase can thus be considered as a first step in the branched pathway for the biosynthesis of several important end products containing C_1 units derived from folate metabolism. 5,-10-CH_2-H_4folate occupies a central position in the pathway for the interconversion of folate coenzymes. It acts as a starting point for the biosyn-

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Abbreviations used: H_2 folate, Dihydrofolate; H_4 folate, tetrahydrofolate; $5\text{-}CH_3\text{-}H_4$ folate, 5-methyltetrahydrofolate; $5,10\text{-}CH_2\text{-}H_4$ folate, 5,10-methylenetetrahydrofolate; $5,10\text{-}CH^+\text{-}H_4$ folate, 5,10-methenyltetrahydrofolate; SDS, sodium dodecyl sulphate; SAM, S-adenosylmethionine; DEAE, diethylaminoethyl.

thesis of thymidilate, purines and formylmethionine-tRNA. The reductase, thymidilate synthetase (EC 2.1.1.45) and 5,10-CH_2-H_4folate dehydrogenase (EC 1.5.1.5) compete for this common intermediate and it is to be expected that mechanisms must be present to channel this intermediate among the competing pathways.

It was suggested that SAM and S-adenosyl homocysteine regulated the activity of this enzyme (Kutzbach and Stockstad, 1971; Daubner and Matthews, 1981). It is also possible that this enzyme activity can be regulated by protein–protein interaction with the other enzymes of the pathway namely thymidilate synthetase, 5,10-CH_2-H_4folate dehydrogenase, H_2folate reductase (EC 1.5.1.3) and serine hydroxymethyltransferase (EC 2.1.2.1). These enzymes are present as protein complexes and also as multifunctional proteins (Paukert *et al.*, 1976; Caperelli *et al.*, 1980). In order to meaningfully understand the regulation of the pathway, it was necessary to isolate all these enzymes from a single source and study their protein–protein and protein-ligand interactions. The dehydrogenase had been isolated from sheep liver (Paukert *et al.*, 1976) and serine hydroxymethyltransferase was recently isolated from the same source, without loss of its regulatory properties (Manohar *et al.*, 1982). In this paper we describe a simple method for the isolation of 5,10-CH_2-H_4 folate reductase from sheep liver and some of its physicochemical and kinetic properties.

Materials and methods

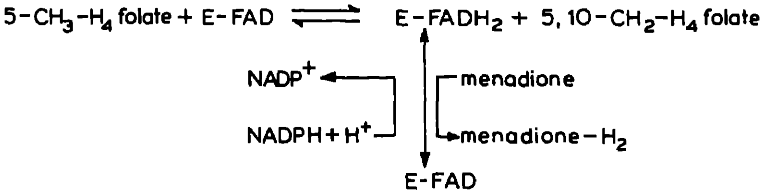
Chemicals

All the chemicals used were of Analytical grade or purchased from Sigma Chemical Co., St. Louis, Missouri, USA, except Blue Sepharose CL6B and DEAE-Sephacel, which were from Pharmacia Fine Chemicals, Uppsala, Sweden. Freund's complete adjuvant and agar were from Difco Laboratories, Detroit, Michigan, USA; H_4folate was prepared by the method of Hatefi *et al.* (1959). [^{14}C]-5-CH_3-H_4folate was from Amersham International Plc, Amersham, UK.

Enzyme assay

The enzyme activity was assayed by three different procedures.

Assay 1: The incubation mixture contained in a final volume of 0.1 ml: 20 μmol of ascorbate, 0.1 μmol FAD, 50 nmol [^{14}C]-5-CH_3-H_4folate (1000 cpm/nmol), 20 μmol potassium phosphate buffer (pH 7.2), 160 nmol EDTA, approximately 70 nmol menadione and an aliquot of the enzyme. The reaction mixture was preincubated at 37° C for 5 min and [^{14}C]-5-CH_3-H_4folate was added with mild agitation to start the reaction. Assay tubes were incubated for 10 min and the reaction was stopped by the addition of 60 μl of freshly prepared dimedone reagent (3 mg/ml dimedone in 1 M acetate buffer, pH 4.5) and heating in a boiling water bath for 5 min. After cooling the assay tubes in ice, the [^{14}C]-labelled formaldehydedimedone adduct was extracted into toluene and the radioactivity measured in a LKB Rack-Beta-Spectrometer. One unit of enzyme activity was defined as the amount of enzyme required to catalyze the formation of 1 μmol of formaldehyde per min. This assay depends on the reoxidation of reduced enzyme generated by the interaction with 5-CH_3-H_4folate by the externally added electron acceptor, menadione according to the following scheme (equation 2).


5-CH_3-H_4 folate + E-FAD
E-FADH$_2$ + 5,10-CH_2-H_4 folate
NADP$^+$
NADPH+H$^+$
menadione
menadione-H$_2$
E-FAD

(2)

This procedure is essentially that described by Donaldson and Keresztesy (1962).

Assay 2: This assay was carried out at 25° C under an atmosphere of nitrogen according to the method of Kutzbach and Stokstad (1971). The assay mixture contained in a total volume of 1 ml: 50 μmol potassium phosphate buffer, pH 7.2; 0.002 μmol FAD; 0.15 μmol NADPH; 10 μmol 2-mercaptoethanol; 1 μmol 5,10-CH_2-H_4 folate and enzyme. The reaction mixture was bubbled with nitrogen, the cuvettes covered with parafilm and the reaction was started after a preincubation period of 5 min, by the addition of either folate substrate, or NADPH with Hamilton gas tight syringe. The initial rates were recorded at 366 nm in Cary 219 recording spectrophotometer.

The activity was expressed as μmol of NADPH oxidized per min. This assay is represented in equation 2.

Assay 3: This assay represents the diaphorase activity of the enzyme. The assay mixture contained in a total volume of 1 ml: 50 μmol potassium phosphate buffer, pH 7.2; 0.002 nmol FAD; 0.15 μmol NADPH and 120 nmol menadione to initiate the reaction. The absorbance change with time was recorded at 343 nm in a Cary 219 recording spectrophotometer.

Electrophoresis

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was carried out at room temperature (23 ± 3° C) in 7.5% gels in Tris-glycine buffer, pH 8.3 containing 0.1% SDS according to the method of Laemmli (1970). The marker proteins used to determine subunit molecular weight were bovine serum albumin (66,000), ovalbumin (45,000), trypsinogen (24,000) and β-lactoglobulin (18,400). The numbers in paranthesis indicate molecular weights (M_r)

Determination of molecular weight by gel filtration

The molecular weight of the native enzyme was determined by the gel filtration (Andrews, 1965) using a Sepharose-4B column (1×60 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.2, $S_{20,w}$ value was determined in a Spinco Model E Analytical Ultracentrifuge using a single sector cell equipped with Schlieren optics and the centrifugation was done at 180,000 *g* at 20° C. The $S_{20,w}$ was calculated according to the method of Schachman (1957).

Immunological techniques

Antiserum to the purified enzyme was raised in a male rabbit weighing approximately 2.5 Kg obtained from the Central Animal Facility of the Institute. The enzyme (500 μg) in Freund's complete adjuvant was injected intradermally at weekly intervals. After 4 weeks, a booster dose of 1 mg of enzyme was directly administered into the thigh muscle. After 9 days, the rabbits were bled through the marginal ear vein and the serum obtained was tested for the presence of antibody. After administering a second booster dose of the enzyme, the animal was bled 9 days later and the serum separated from the blood clot was used as the antiserum after partial purification by $(NH_4)_2SO_4$ precipitation and passage through diethylaminoethyl (DEAE)-cellulose chromatography.

Ouchterlony double diffusion analysis was carried out in an agar plate (Ouchterlony, 1958). Antiserum (100 μl) was placed in the central well and 50 μg of enzyme was placed in the surrounding two wells.

Results

Isolation and purification of the 5,10-CH_2-H_4folate reductase from sheep liver

Potassium phosphate buffer, 0.05 M, pH 7.2 containing 0.3 mM EDTA (designated as Buffer A) was used extensively in the study and all the steps of purification were carried out at 0–5° C.

Fresh liver obtained from healthy sheep immediately after slaughter was chilled in ice and transported quickly from the local abattoir to the laboratory. The liver (125 g) was trimmed of excess fat, connective tissue and gall bladder. The liver was cut into slices and homogenized in 400 ml of Buffer A using a Waring blendor at high speed for 1.5 to 2 min. The pH of the homogenate was adjusted to 6.0 by adding 4 M acetic acid and centrifuged at 27,000 *g* for 10 min. To the supernatant, solid ammonium sulphate was added to 25% saturation, stirred for 10 min and centrifuged at 27,000 *g* for 10 min. The supernatant fraction was raised to 50% saturation by a further addition of solid ammonium sulphate. The precipitate obtained after centrifugation at 27,000 *g* was suspended in cold distilled water in a Potter-Elvehjem homogenizer. The pH of this suspension was adjusted to 4.5 using 4 M acetic acid. The precipitate was dissolved in Buffer A and the pH adjusted to 7.2 with 1 M NH_4OH and was left overnight. After centrifugation, the supernatant was designated as "acid precipitate" (table 1). This fraction was diluted with an equal volume of Buffer A and loaded at a flow rate of 30 ml/h on to a DEAE-Sephacel column (2.5 × 40 cm) equilibrated with Buffer A containing 10% glycerol. The column was washed with 4 bed volumes of Buffer A containing 0.1 M KCl and 10% glycerol until the absorbance of the eluate at 280 nm was less than 0.05. The column was washed further with one bed volume of Buffer A containing 0.3 M KCl and 10% glycerol and the enzyme was eluted with 2 bed volumes of 0.6 M KCl in Buffer A. Active fractions were pooled, diluted to twice the original volume and loaded at a flow rate of 15 ml/h on to a Blue Sepharose column (2.5 × 10 cm), equilibrated with Buffer A containing 0.3 M KCl and 10% glycerol. The column was washed with 2–3 bed volumes of equilibrating buffer and the enzyme was eluted from the column with 2 bed volumes of 2 M KCl in Buffer A containing 10% glycerol. Active fractions were pooled and concentrated in an Amican ultrafiltration cell using XM-50 membrane. Results of a typical purification procedure are summarised in table 1.

Table 1. Summary of purification procedure for 5,10-CH_2-H_4 folate reductase from sheep liver.

Step	Protein (mg)	Activity units*	Specific activity $\times 10^3$	Fold purification	Recovery
$(NH_4)_2SO_4$ fractionation	3,000	39.0	13	1	100
Acid precipitation	834	38.2	45	4	98
DEAE-Sephacel chromatography	53	8.8	166	13	22
Blue Sepharose affinity chromatography	6	7.8	1305	100	20

* μmol HCHO formed/min at 37° C.

The procedure yielded 6 mg of the enzyme with a specific activity of 1–1.3 units/mg protein. From the $(NH_4)_2SO_4$ fractionation step, the fold purification of the enzyme was 100 and the recovery was 15–20%. The activity could not be detected in the crude extract possibly due to the presence of the enzyme in low amounts or the occurrence of an endogenous inhibitor. The enzyme in the acid precipitation steps yielded considerable degree of purification. The enzyme was strongly bound to Blue Sepharose, suggesting the presence of nucleotide binding site (Stellwagen, 1977). Dihydrofolate reductase (Subramanian and Kaufman, 1980) and serine hydroxymethyltransferase (Ramesh and Appaji Rao, 1980; Manohar *et al.*, 1982) also contain binding sites for this dye at or near their active sites. These observations suggest similarities in the topology of the active sites of enzymes of folate metabolism and is not surprising in view of the structural similarities between this triazine portion of the dye and the pteridine moiety of folic acid.

Stability of the enzyme

The enzyme prepared without glycerol was very unstable and lost all the activity within 5–6 h. However, inclusion of 10% glycerol in all buffers used during and after acid precipitation step stabilised the enzyme for 2 weeks when stored at −40° C.

Criteria of homogeneity

(1) SDS-Polyacrylamide gel electrophoresis of the enzyme preparation (50 μg) gave a single band on 7.5% gel (figure 1A); (2) Upon ultracentrifugation, the enzyme sedimented as a single sharp symmetrical peak. The sedimentation coefficient value ($S_{20,w}$) was calculated to be 11; (3) Ouchterlony immunodiffusion test gave a single precipitin line and the ends of the precipitin lines joined without spur formation (figure 1B); (4) Enzyme was eluted as a single symmetrical peak on gel filtration in a calibrated Sepharose-4B column (figure not given).

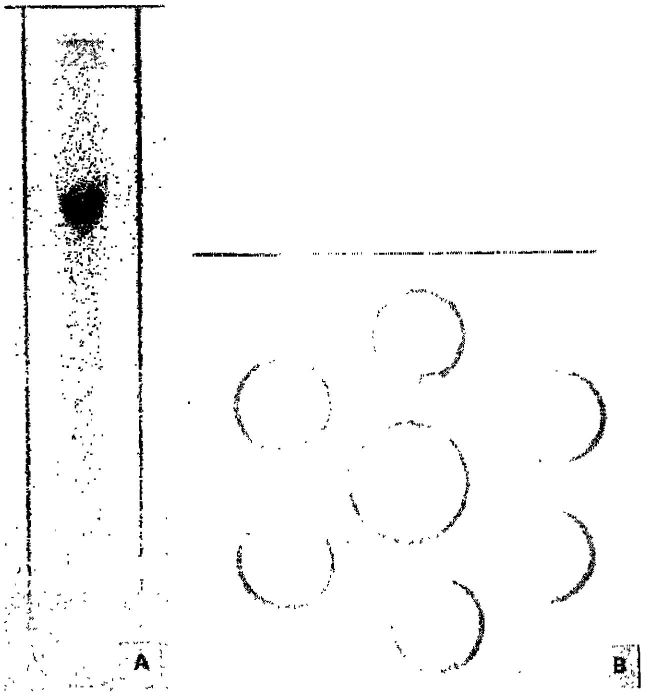


Figure 1. A. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the purified enzyme in 7.5% gel. The enzyme (250 μg) was treated with sodium dodecyl sulphate (2%) and heated for 3 min at 100° C. An aliquot corresponding to 40 μg protein was subjected to electrophoresis at a current of 4 mA/tube. The gel was stained with Coomassie Brilliant Blue R and destained with a mixture of methanol and acetic acid and water. **B.** Ouchterlony immunodiffusion analysis of sheep liver 5,10-CH_2-H_4 folate reductase. Antiserum was placed in the central well and 50 μg of the enzyme was placed in the surrounding two wells.

Determination of molecular weight and subunit composition of the enzyme

The apparent M_r of the native enzyme estimated by gel filtration using a calibrated Sepharose-4B column was 1,66,000 ± 5,000 (figure 2). Subunit molecular weight was determined by SDS-polyacrylamide gel electrophoresis to be 87,000 ± 5,000 (figure 2 inset) suggesting that the enzyme was probably a dimer.

Catalytic properties of the enzyme

The specific activity of the enzyme isolated by the above procedure was 1.2 units/mg protein (one unit activity is defined as the amount of enzyme required to form 1 μmol formaldehyde per mg protein per min). Assuming the existence of two identical sites, the turnover number per catalytic site was calculated to be 208 min^{-1}. When the enzyme was preincubated with menadione and the reaction was started with 5-CH_3-H_4 folate, activity increased with increasing concentration

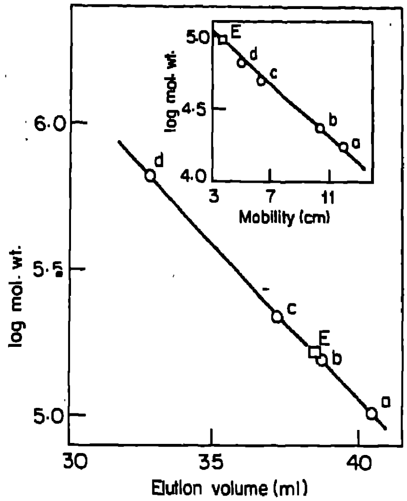


Figure 2. Determination of molecular weight and subunit composition of sheep liver 5,10-CH_2-H_4 folate reductase.

For the determination of molecular weight, the enzyme (E) was passed through a Sepharose-4B column. The standard protein markers used were (a) hexokinase; (b) glucose oxidase; (c) catalase; and (d) thyroglobulin. The elution volumes were plotted as a function of log molecular weight. The molecular weight of 5,10-CH_2-H_4 folate reductase was determined to be 1,66,000±5,000. Inset represents sodium dodecyl sulphate-polyacrylamide slab gel electrophoresis in 7.5% gel. The enzyme (250 μg) and the marker proteins (250 μg) were separately subjected to denaturation by sodium dodecyl sulphate as described in figure 1A. The electrophoresis, staining and destaining were done as described in figure 1A. The mobilities of (a) β-lactoglobulin; (b) trypsinogen; (c) ovalbumin; (d) bovine serum albumin were plotted as a function of log molecular weight. The subunit molecular weight of the enzyme (E) was determined by extrapolation to be 87,000±5,000.

of enzyme upto 7 μg/ml (figure 3). The time course of this reaction showed an initial burst when it was started by the addition of menadione after preincubating the enzyme with 5-CH_3-H_4 folate for 5 min. However, the reaction rate was linear upto 10 min when the reaction was started with 5-CH_3-H_4 folate after preincubation with menadione (figure 4A, B). When the temperature of the reaction was varied from 20° C to 90° C, an optimum temperature of 37° C was observed with a complete loss of activity beyond 50° C. The maximum activity was observed at pH 6.5, which is in good agreement with the pH optimum for the enzyme reported from other sources (Kutzbach and Stokstad, 1971). The enzyme exhibited three activities: (a) 5-CH_3-H_4 folate—menadione oxidoreductase (equation 2); (b) NADPH—menadione oxidoreductase; (c) NADPH—5,10-CH_2-H_4 folate oxido-reductase activities (equation 2). Menadione was a better electron acceptor than dichlorophenol indophenol and cytochrome-C (table 2).

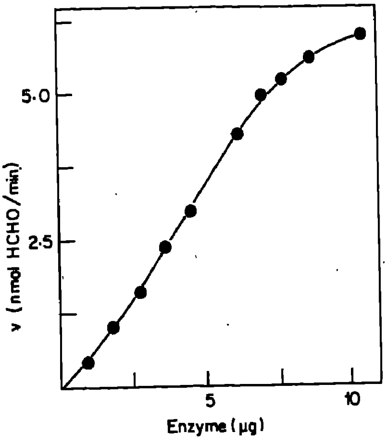


Figure 3. Enzyme concentration curve. Different concentrations of enzyme were preincubated for 5 min at 37° C with menadione (1 mM), and other assay components and the reaction was started with 5-CH_3-H_4folate (0.5 mM). The activity was measured as described in assay 1.

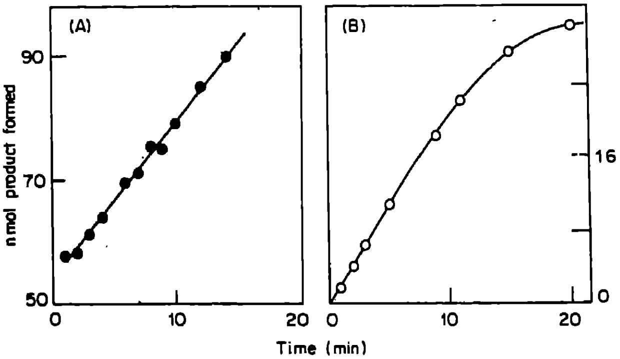


Figure 4. Time course of the reaction catalysed by 5-10-CH_2-H_4folate reductase monitored using assay 1. **A.** Enzyme was preincubated with 5-CH_3-H_4 folate and other assay components for 5 min at 37° C. The reaction was started with menadione and was stopped at various time intervals (●). **B.** Enzyme was preincubated with menadione and other assay components for 5 min at 37° C. The reaction was started with 5-CH_3-H_4folate and was stopped at different time intervals (O).

Table 2. Relative electron acceptor activities in the radioactive assay of 5,10-CH_2-H_4folate reductase.

Electron acceptor	Activity (nmol)
Menadione	2.8
O_2	2.1
Dichlorophenol indophenol	1.0
Cytochrome-C	1.6

The enzyme showed a hyperbolic saturation pattern for the 5-CH_3-H_4folate-menadione oxidoreductase activity when initial velocity was plotted against varying concentrations (0.05–0.5 mM) of 5-CH_3-H_4folate (figure 5). Lineweaver-Burk plot of this saturation data was linear (figure 5 inset). $K_{0.5}$ value of 0.13 mM

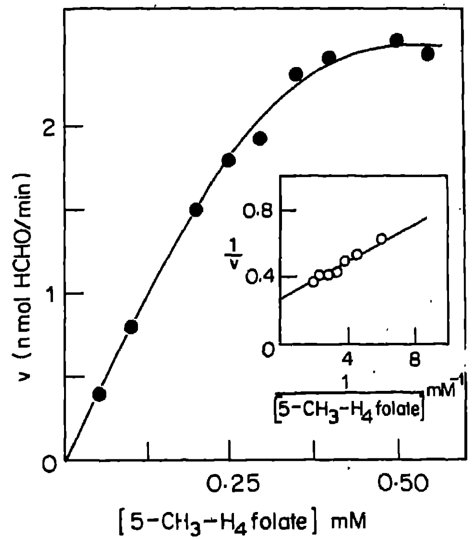


Figure 5. The 5-CH_3-H_4 folate saturation pattern of the enzyme in assay 1. Enzyme was preincubated with menadione and other assay components for 5 min at 37° C and the reaction was started with various concentrations of 5-CH_3-H_4 folate (0.05–05 mM) as a mixture of cold and hot 5-CH_3-H_4folate. The inset shows the Lineweater-Burk plot.

was calculated from the Hill plot. When menadione (5–200 μM) and NADPH (10–150 μM) were varied at saturating concentrations of the other substrate in the NADPH-menadione reductase assay, a hyperbolic saturation pattern was obtained (figure 6A, B). The $K_{0.5}$ values for menadione and NADPH were calculated to be 2.45 μM and 16 μM, respectively from the Hill plots.

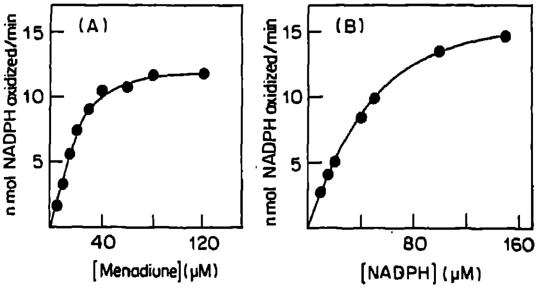


Figure 6. A. Saturation pattern for menadione in menadione reductase assay. After preincubating the enzyme with 150 μM NADPH at room temperature, the reaction was started with varying concentrations of menadione (5–200 μM). **B.** Saturation pattern for NADPH in menadione reductase assay. After preincubating the enzyme with varying concentrations of NADPH (10–150 μM), the reaction was started with saturating concentration of menadione (125 μM).

Kinetic mechanisms

To establish the kinetic mechanism, NADPH-menadione reductase assay was used. Evidence for a ping-pong mechanism was obtained from initial velocity and product inhibition studies. Double reciprocal plots of the initial velocities when menadione concentration was varied at different fixed concentrations of NADPH (16, 50 and 150 μM) gave parallel set of lines as shown in figure 7A. Similar pattern was observed when NADPH was varied at different fixed levels of menadione (60, 120 μM) (figure 7B). The parallel set of lines obtained in both the cases suggest that a ping-pong mechanism may be operating. This was further supported by three experiments using product as an inhibitor. In the first experiment, the NADPH concentration was varied (8–40 μM) at different fixed concentrations (4, 6 and 8 mM) of the product NADP at unsaturating (30 μ M) menadione concentration and the double reciprocal plots gave a series of intersecting lines indicating non-competitive type of inhibition (figure 8A). In the second experiment, when

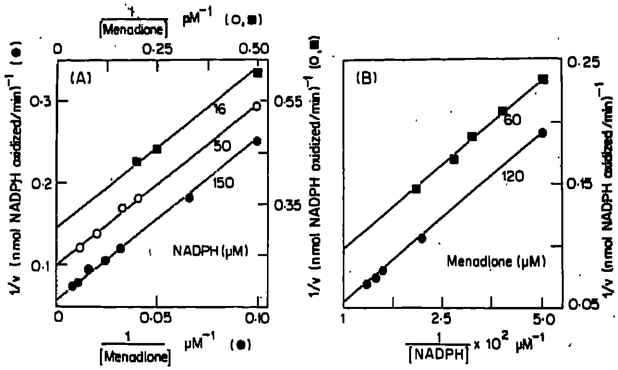


Figure 7. A. Double reciprocal plots for menadione saturation at different fixed concentrations of NADPH. Menadione saturation patterns were done at different concentrations of NADPH i.e., 16 μM (■), 50 μM (O), 150 μM (●). **B.** Double reciprocal plots for NADPH saturation at different fixed levels of menadione. NADPH saturations were done at two different concentrations of menadione i.e, 60 μM (■), 120 μM (●).

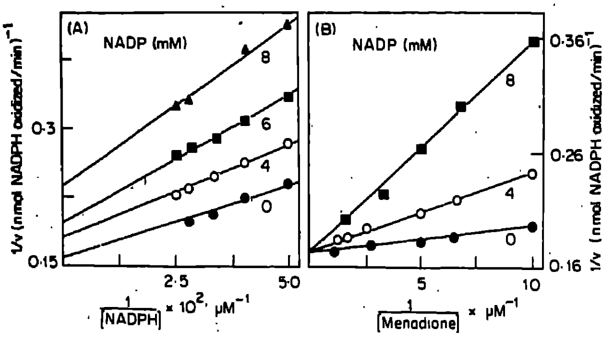


Figure 8. Product inhibition analysis by NADP. **A.** Non-competitive inhibition by NADP with NADPH. NADPH saturation patterns were done at different fixed concentrations of NADP, i.e, 0 mM (●), 4 mM (O), 6 mM (■), 8 mM (▲), and at 30 μM menadione. **B.** Competitive inhibition by NADP with respect to menadione. Menadione saturation patterns were done at 50 μM NADPH and at different concentrations of NADP i.e., 0 mM (●), 4 mM (O), 8 mM (■).

NADPH was varied at 10 mM NADP (the concentration at which 75% of inhibition was observed at unsaturating (60 μM) menadione concentration) and at a saturating (125 μM) menadione concentration, no inhibition was observed. In the third experiment, menadione was varied (10–120 μM) at different fixed concentrations (4, 8 mM) of NADP and at unsaturating (50 μM) NADPH concentration. A set of lines intersecting on the ordinate was observed indicating competitive inhibition by NADP (figure 8B).

Discussion

It was earlier reported that SAM regulated the activity of 5,10-CH_2-H_4 folate reductase from pig liver (Kutzbach and Stokstad, 1971; Daubner and Matthews, 1981). However, other mechanisms of regulation of this enzyme and its kinetic mechanism were not investigated. Based on the results presented in this paper, the following kinetic mechanism for the reaction catalyzed by 5,10-CH_2-H_4 folate reductase can be postulated (figure 9).

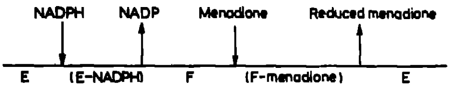


Figure 9. Ping-pong mechanism for the diaphorase activity catalyzed by the 5,10-CH_2-H_4 folate reductase.

The parallel set of lines obtained when menadione or NADPH concentrations were varied at different fixed concentrations of the second substrate suggested that a ping-pong mechanism could be operative for the reaction catalysed by this enzyme (figure 7A, B). In product inhibition studies, when NADPH was varied at a fixed subsaturating concentration of menadione and at different fixed concentrations of NADP (figure 8A), a set of intersecting lines were obtained showing that NADP was a non-competitive inhibitor with respect to NADPH. Such a pattern could arise if NADPH and NADP are binding to two different forms of the enzyme (E and F, respectively) that are linked by reversible steps. In the second experiment, when NADPH was varied at saturating concentration of menadione and at 10 mM NADP, no inhibition was observed. The absence of inhibition is due to menadione and NADP binding to the same form of enzyme and enzyme is not available for NADP interaction at saturating menadione concentration. The competitive inhibition observed, when menadione was varied at subsaturating concentration of NADPH and at different fixed concentrations of NADP supports this observation. Using the rules enunciated by Cleland (1970), the substrate saturation and product inhibition pattern for the mechanism given in figure 9, were predicted and compared with the observed pattern (table 3). All these results are compatible with the ping-pong mechanism postulated in figure 9.

Table 3. Comparison of the observed initial velocity and product inhibition patterns for the reaction catalysed by 5,10-CH_2-H_4 folate reductase.

	Predicted patterns			
Property	Ordered	Random	Ping-pong	Observed patterns
Initial velocity: Menodine varied. NADPH different fixed concentrations.	Intersecting lines	Intersecting lines	Parallel lines	Parallel lines
NADPH varied. Menadione different fixed concentrations	Intersecting lines	Intersecting lines	Parallel lines	Parallel lines
Product inhibition: NADPH varied. Menadione unsaturated	Non-competitive	Non-competitive	Non-competitive	Non-competitive
NADPH varied. Menadione saturated	Un-competitive	Non-competitive	No inhibition	No inhibition
Menadione varied. NADPH unsaturated.	Non-competitive	Non-competitive	Competitive	Competitive

The reaction shown above is a diaphorase assay to measure the activity of the enzyme and the physiological reaction is the pyridine nucleotide dependent reduction of 5,10-CH_2-H_4 folate. By analogy with diaphorase reaction the reduction of 5,10-CH_2-H_4 folate can be postulated to occur by the following kinetic mechanism (figure 10).

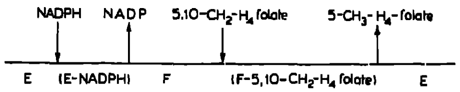


Figure 10. Kinetic mechanism for the reduction of 5,10-CH_2-H_4 folate by NADPH.

Evidence to suggest that 5-CH_3-H_4 folate reacts with the E form of enzyme was obtained by preincubating the enzyme with 5-CH_3-H_4 folate prior to starting the reaction by the addition of menadione in the radioactive assay. A burst in the reaction velocity was observed (figure 4A). The burst could be attributed to the reduction of the enzyme by 5-CH_3-H_4 folate to form reduced enzyme and 5,10-CH_2-H_4 folate which dissociated to liberate formaldehyde. Further evidence to support this mechanism can be obtained by spectrally monitoring the reduction of this enzyme by NADPH and the 5-CH_3-H_4 folate and determining the product inhibition and the substrate saturation patterns of the physiological reaction.

The elucidation of the kinetic mechanism of the reaction catalysed by this enzyme and the availability of the enzyme in a purified form now enables a detailed study of the regulation of this enzyme activity by interaction with nucleotides and other enzymes of the pathway as well as the changes in its activity in neoplastic tissues.

Acknowledgement

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Effect of sodium dodecyl sulphate on the high molecular weight protein fraction of mustard (*Brassica juncea*) and rapeseed (*Brassica campestris*)

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Abstract. The effect of sodium dodecyl sulphate on mustard and rapeseed 12 S protein has been monitored by the techniques of ultracentrifugation, viscosity, difference spectra and fluorescence spectrophotometry. At low concentration of sodium dodecyl sulphate (<3.47 mM) mustard protein undergoes aggregation and at higher concentrations it dissociates to 1.8 S protein, the dissociation being complete at 17.3 mM sodium dodecyl sulphate. The rapeseed protein, on the other hand, undergoes dissociation at all the concentrations of sodium dodecyl sulphate. The reduced viscosity values of mustard protein in the presence of the denaturant are higher than those of rapeseed protein. Similarly in difference spectra change in absorbance values of mustard protein are higher. The relative fluorescence intensity of the mustard protein increases with sodium dodecyl sulphate concentration, upto 0.87 mM and this is followed by fluorescence quneching at higher denaturant concentrations. However, with the rapeseed protein fluorescence quenching was observed at all concentrations of sodium dodecyl sulphate.

Keywords. Mustard protein; rapeseed protein; denaturation; sodium dodecyl sulphate.

Introduction

We have reported that the high molecular weight (mol. wt.) protein fraction, the 12 S protein, from mustard (*Brassica juncea*) and rapeseed (*Brassica campestris*) differ in amino acid composition, subunit composition, susceptibility to proteolysis and secondary structure (Gururaj Rao and Narasinga Rao, 1981). They also differ in their susceptibility to denaturation by urea and guanidinium hydrochloride (GndHCl); rapeseed protein is more easily denatured (Gururaj Rao and Narasinga Rao, 1983). In this communication we report the results of the effect of sodium dodecyl sulphate (SDS) on the mustard and rapeseed 12 S protein.

Materials and methods

Materials

The source of mustard seed and rapeseed was the same as described previously (Gururaj Rao and Narasinga Rao, 1983). SDS from British Drug House, India was recrystallised from 50% ethanol.

Abbreviations used: SDS, Sodium dodecyl sulphate; GndHCl, guanidinium hydrochloride; RTIC, rotor temperature indicator and control; mol. wt., molecular weight.

Isolation of the 12 S protein

It was isolated by the procedure reported earlier (Gururaj Rao and Narasinga Rao, 1981).

Protein concentration

This was determined by absorbance measurements at 280 nm, using a value of $E_{1\,\text{cm}}^{1\%}$: 9.9 for mustard protein and 10.8 for rapeseed protein, (Gururaj Rao, 1980).

Ultracentrifugation was performed at room temperature (~27° C) in a Spinco Model E analytical ultracentrifuge equipped with rotor temperature indicator and control (RTIC) unit and phase plate schlieren optics, using 1% protein solution in the appropriate solvent. The solution was left at room temperature for 3 h after the addition of SDS. The runs were made at 59,780 rpm. From the photographs taken at different intervals of centrifugation, $S_{20\,w}$ was calculated by the standard procedure (Schachman, 1959). Weight-average sedimentation coefficient, $\bar{S}_{20,w}$, was calculated from the second moment of the entire gradient curve (Goldberg, 1953).

Gel filtration

Sephadex G-200 (Pharmacia, Sweden), which had been equilibrated with the buffer, was packed into a column 2.5×50 cm. Protein solution (5 ml) containing about 50 mg of protein was loaded on the column. When the experiment was performed in the presence of SDS, the column was equilibrated with the buffer containing the appropriate concentration of SDS and the eluting buffer also contained SDS. Fractions (4.0 ml) were collected and absorbance measured at 280 nm.

Viscosity

The measurements were made at 30±0.1° C in an Ostwald viscometer having a flow time of 184 *s* with distilled water. Protein (1%) solution was used. The blanks for these measurements contained the appropriate concentration of SDS.

Difference spectra

Difference spectra produced by the addition of different amounts of SDS to the protein were recorded in a Perkin-Elmer double beam recording spectrophotometer, 124, in the range 240 to 300 nm. Protein solutions having an absorbance of 1.8 at 280 nm were used.

Fluorescence spectra

A Perkin-Elmer fluorescence spectrophotometer Model 203 was used. The emission was measured in the range 300–400 nm after excitation at 280 nm. Protein solution having an absorbance of 0.05 at 280 nm was used.

Other measurements

A Radiometer pH meter TTT2 was used for pH measurements and a Carl-Zeiss ultra-violet spectrophotometer for absorbance measurements.

Results and discussion

Unless otherwise mentioned all the measurements were made in 0.025 M Tris-glycine buffer of pH 8.3.

The sedimentation velocity pattern of the mustard protein consisted of a single symmetrical peak with an $S_{20,w}$ value of 11.3 (figure 1A). The addition of SDS upto 0.87 mM did not cause any change in either the pattern or the sedimentation coefficient. At 1.21 mM SDS, however, the peak became less symmetrical and broad.

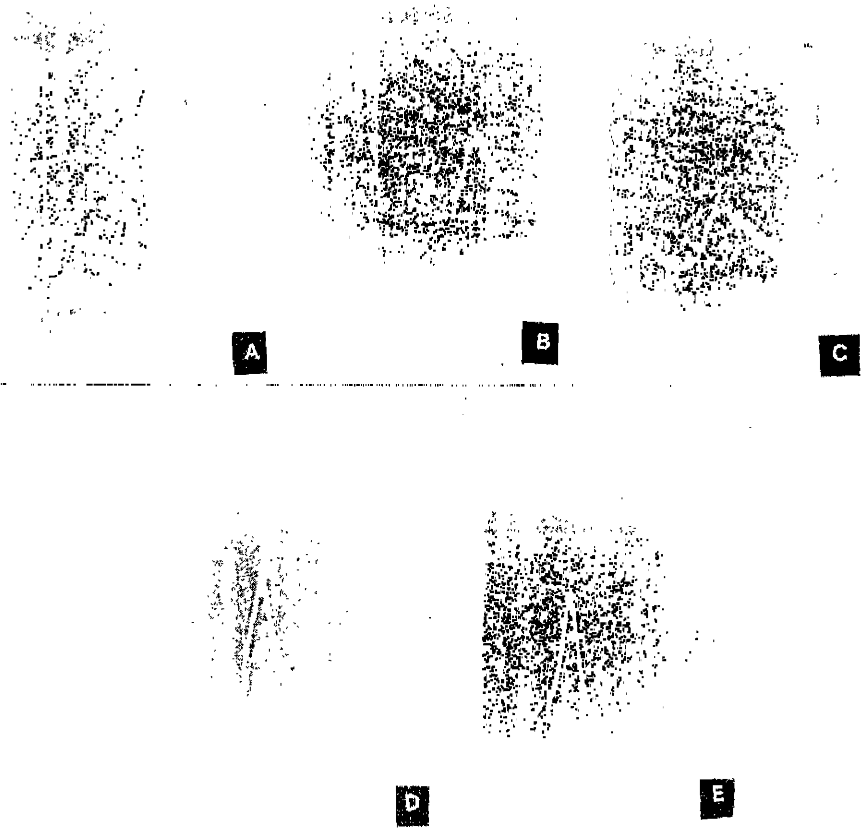

Figure 1. Sedimentation velocity pattern of mustard 12 S protein (in 0.025 M Tris-glycine buffer, pH 8.3).

A. Protein; **B.** Protein + 1.21 mM SDS; **C.** Protein + 3.47 mM SDS; **D.** Protein + 8.67 mM SDS; **E.** Protein + 17.34 mM SDS.

This effect was more pronounced at higher concentrations of SDS up to 3.47 mM. The $S_{20,w}$ value increased with SDS concentration; at 3.47 mM it attained a value of 14.0 S (figure 2). At higher concentrations, dissociation into 1.8 S protein occurred (figure 1D) and at 17.34 mM SDS concentration, 1.8 S protein alone was present (figure 1E).

The effect of SDS on the rapeseed protein was different. It dissociated the 12 S protein into 1.8 S protein even at low concentration and dissociation was complete at 17.34 mM SDS (figure 3). No evidence for any assymetrical peak nor increase in $S_{20,w}$ was observed. $S_{20,w}$ value decreased continuously with increase in SDS concentration (figure 2).

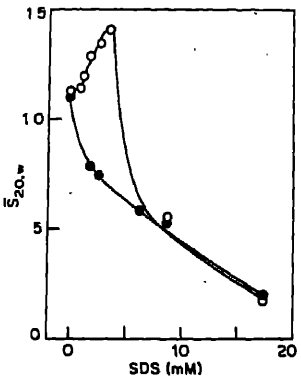

Figure 2. Weight-average sedimentation coefficient, $S_{20,w}$ as a function of SDS concentration.

(○), Mustard 12 S protein; (●), rapeseed 12 S protein.

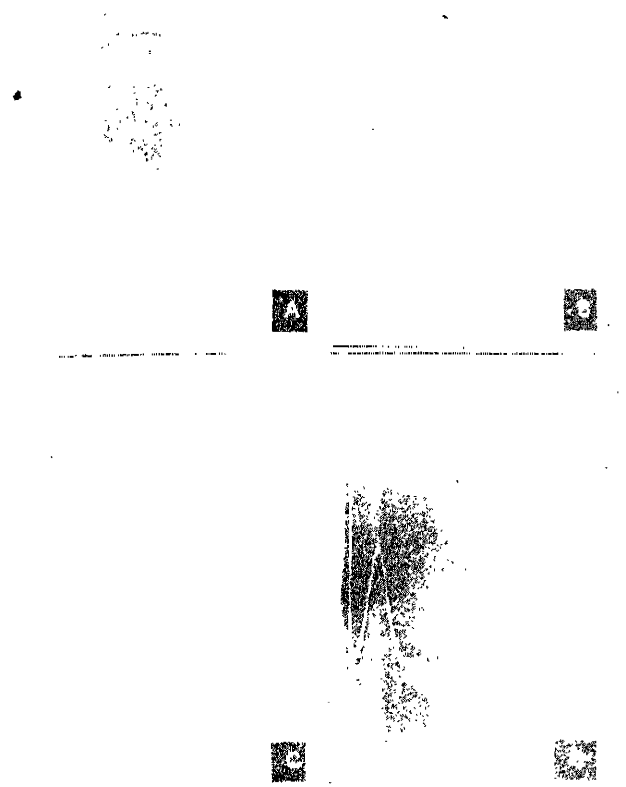

Figure 3. Sedimentation velocity pattern of rapeseed 12 S protein (in 0.025 M Tris-glycine buffer pH 8.3).

A. Protein; **B.** Protein + 2.60 mM SDS; **C.** Protein + 3.47 mM SDS; **D.** Protein + 17.34 mM SDS.

In gel filtration experiments mustard 12 S protein gave a major peak eluting at 230 ml (figure 4A). The latter is a non-protein fraction (MacKenzie and Blakely, 1972). With the addition of SDS, the elution volume of the major peak shifted to lower value, and at 2.6 mM SDS, it was 88 ml. In our experiments, the accuracy of determination of elution volume (V_e) is expected to be ±4 ml. Shift of V_e to lower value would suggest aggregation of the protein. At higher SDS concentrations, there were fractions eluting at 110 and 140 ml suggesting dissociation of the protein (figure 4C).

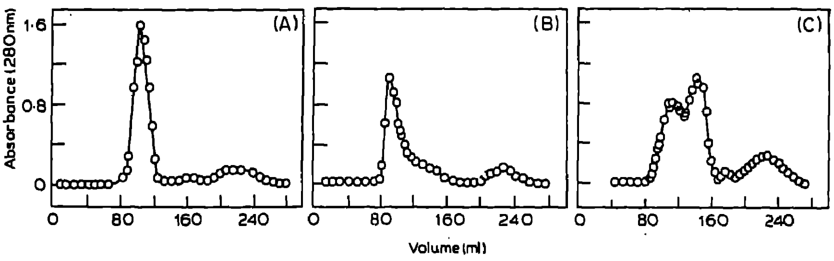

Figure 4. Gel filtration pattern of mustard 12 S protein on Sephadex G-200 (in 0.025 M Tris-glycine buffer, pH 8.3).
A. Protein; **B.** Protein + 2.60 mM SDS; **C.** Protein + 8.67 mM SDS.

In the case of the rapeseed protein association of the protein was not indicated at any SDS concentration. At 2.6 mM SDS concentration, V_e of the major peak was the same as that of the protein in the absence of SDS (figure 5A, B). However the peak was less sharp and a shoulder at 125 ml was observed. At 8.7 mM SDS the major peak had shifted to 110 ml and another peak at 132 ml was observed.

Thus both ultracentrifugation and gel filtration experiments indicated that at low concentrations of SDS mustard protein aggregated to a high mol. wt. species and at high concentrations it dissociated the protein to low mol. wt. (1.8 S) protein. On the other hand rapeseed protein showed only dissociation.

Reduced viscosity of the mustard protein was 5.3 cc/g. Addition of SDS increased it (figure 6). The increase was in two steps; upto 5,20 mM SDS it increased and attained a constant value between 5.20 and 10.40 mM SDS, then increased and attained a steady value of 11.5 cc/g. Thus even at the highest SDS concentration, the protein did not attain random-coil conformation (Tanford, 1970). Rapeseed protein had a value of 4.4 cc/g for reduced viscosity. Addition of SDS increased the value. There was no clear indication of two steps. The higest value obtained at 17.34 mM SDS was 10.4 cc/g, slightly lower than with mustard protein.

The addition of SDS produced difference spectra with troughs at 295 nm, 287 nm and 278 nm. The intensity of the troughs increased with SDS concentration (figure 7). This was true of both the proteins. The ΔA values of the mustard protein (figure 7A) at 287 nm or 295 nm at any SDS concentration, were higher than those of rapeseed protein (figure 7B) at the same SDS concentration, suggesting greater perturbation of the tyrosine residues of the mustard protein.

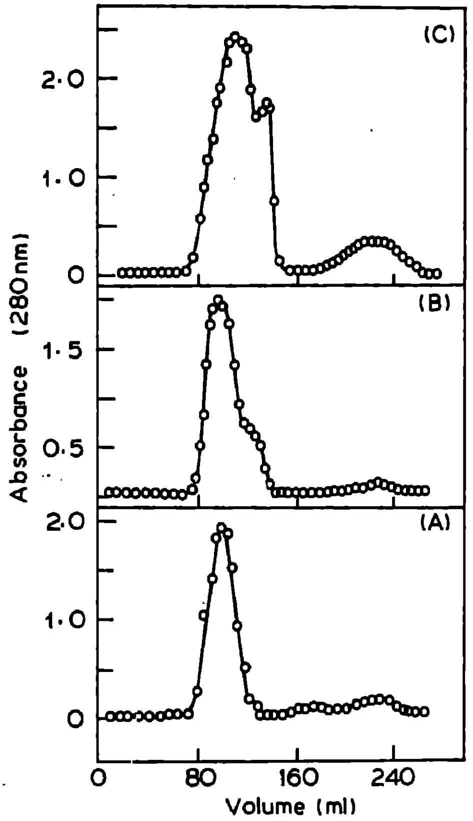

Figure 5. Gel filtration pattern of rapeseed 12 S protein on Sephadex G-200 (in 0.025 M Tris-glycine buffer, pH 8.3).

A. Protein; **B.** Protein + 2.60 mM SDS; **C.** Protein + 8.67 mM SDS.

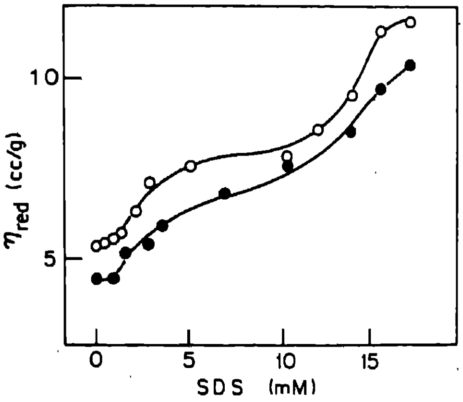

Figure 6. Reduced viscosity of the protein as a function of SDS concentration (in 0.025 M Tris-glycine buffer, pH 8.3).

(O), Mustard 12 S protein; (●), rapeseed 12 S protein.

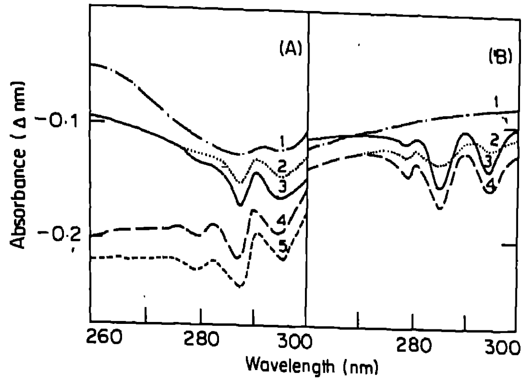

Figure 7. Difference spectra of the protein (in 0.025 M Tris-glycine buffer, pH 8.3).
A. Mustard 12 S protein.
1, Protein + 1.73 mM SDS; 2, Protein + 2.60 mM SDS; 3, Protein + 3.47 mM SDS; 4, Protein + 6.94 mM SDS; 5, Protein + 17.34 mM SDS.
B. Rapeseed 12 S protein.
1, Protein + 1.73 mM SDS; 2, Protein + 2.60 mM SDS; 3, Protein + 6.94 mM SDS; 4, Protein + 17.34 mM SDS.

The addition of SDS caused changes in the fluorescence emission spectrum of the proteins. In the case of mustard protein, SDS upto 1.73 mM caused an *increase* in the emission intensity, then decreased it and a plateau value was reached between 5 and 17 mM SDS (figures 8A and 9). Further there was also a red shift in the emission maximum at higher SDS concentration. On the other hand with the rapeseed protein there was fluorescence quenching at all SDS concentrations (figure 9B); plateau value was reached between 5 and 17 mM SDS. Here also a red shift was observed at higher SDS concentration.

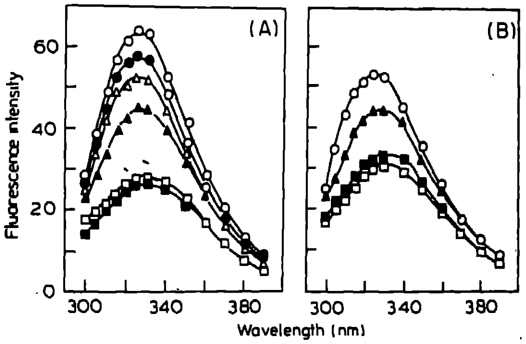

Figure 8. Fluorescence spectra of the proteins (in 0.025 M Tris-glycine buffer, pH 8.3).
A. Mustard 12 S protein.
(○), Protein; (●), Protein + 0.35 mM SDS; (△), Protein + 0.87 mM SDS; (▲), Protein + 1.73 mM SDS; (□), Protein + 8.67 mM SDS; (■), Protein + 17.34 mM SDS.
B. Rapeseed 12 S protein.
(○), Protein; (▲), Protein + 1.73 mM SDS; (■), Protein + 8.67 mM SDS; (□), Protein + 17.34 mM SDS.

Increase in reduced viscosity, appearance of difference spectra and fluorescence quenching (and red shift of emission maximum) can all be taken as evidence of denaturation of the proteins by SDS. Quantitatively speaking, denaturation of the

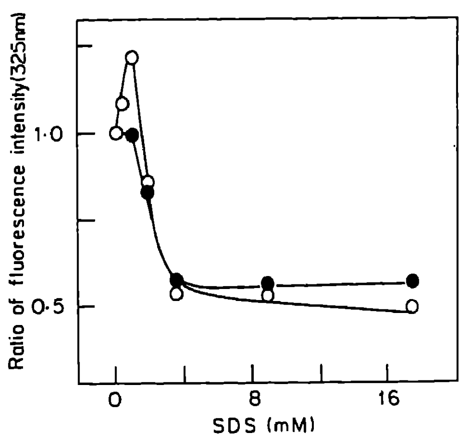

Figure 9. Ratio of fluorescence intensity at 325 nm as a function of SDS concentration.
(O), Mustard 12 S protein; (●), rapeseed 12 S protein.

mustard protein occurred to a greater extent than that of the rapeseed protein, as indicated by viscosity and difference spectral data.

These measurements indicate that SDS causes both dissociation and denaturation of the mustard and the rapeseed 12 S protein. However there were some significant differences in the effect of SDS on the two proteins. These were (a) at low concentrations (<3.5 mM) SDS caused aggregation of the mustard 12 S protein and this was followed by dissociation to 1.8 S protein; however with the rapeseed 12 S protein dissociation occurred at all SDS concentrations; (b) the increase in the reduced viscosity of the mustard protein with SDS concentration followed a clearly delienated two-step process, whereas with the rapeseed protein it was not so clear; and (c) the emission fluorescence intensity of the mustard protein increased upto 0.87 mM SDS and then there was fluorescence quenching; with the rapeseed protein quenching occurred at all SDS concentrations.

Aggregation of proteins by SDS has been reported (Ray and Chatterjee, 1967; Jones and Wilkinson 1976; Jones and Rumby, 1978). It is possible that SDS anions first bind to the available cationic sites on the molecule causing an unfolding and exposure of the hydrophobic groups. Aggregation could then occur through hydrophobic interaction. Burkhard and Stolzenberg (1972) report that the interaction of SDS with ferricytochrome C at pH 7.35 occurs through a preliminary electrostatic interaction followed by interaction between the hydrophobic amino acid residues of the protein.

The increase in the fluorescence intensity of the mustard protein at low concentration of SDS could be due to the aggregation of the protein at these SDS concentrations. Fluorescence is due to the imbedded tyrosine and tryptophane residues (Teale, 1960). Aggregation of the protein would make more groups imbedded and this would increase fluorescence intensity. Reference to figures 2 and 9 would show that $S_{20,w}$ reached the maximum value of 14.0 S at 3.7 mM SDS, whereas the maximum increase in fluorescence intensity occurred at 1 mM. This could possibly be due to the effect of protein concentration; for ultracentrifugation 1% protein solution was used and for fluorescence measurements it was 0.005%.

Apparently the denaturation of the mustard 12 S protein by SDS followed a two-step process as indicated by viscosity data. Several proteins follow more than a single-step process in their denaturation (Tanford, 1970).

The reason for the different effect of SDS on the two proteins is not obvious. Proteins bind SDS avidly (Reynolds and Tanford, 1970). The binding would be by ionic and hydrophobic bonds (Steinhardt and Reynolds, 1969). Although there is some difference in the amino acid composition of mustard and rapeseed 12 S protein (Guraraj Rao and Narasinga Rao, 1981) the hydrophobicity value per residue or the fractional charge per residue of the two proteins does not differ signficantly (Gururaj Rao and Narasinga Rao, 1983). In spite of this there was some difference in the binding of SDS by the two proteins, as suggested by the following experiments.

At low concentrations of SDS upto 3.47 mM aggregation of the mustard protein occurred where as dissociation of the rapeseed protein occurred. When the protein solution containing SDS was dialysed extensively against the corresponding concentration of SDS and analysed in the ultracentrifuge, the mustard protein did not show aggregation, contrary to what was observed with the undialysed solution; actually dissociation occurred. In the case of the rapeseed protein identical results were obtained with the dialysed and undialysed solutions. Obviously in the undialysed solution SDS became limiting because of avid binding by the protein. Since dialysis did not make any difference in the case of rapeseed proten, it would imply that it binds SDS less avidly than the mustard protein. The difference in binding of SDS could possibly be due to differences in the sub-unit composition of the two proteins (Gururaj Rao and Narasinga Rao, 1981).

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Denaturation of the high molecular weight protein fraction of mustard (*Brassica juncea*) and rapeseed (*Brassica campestris*) by urea or guanidinium hydrochloride

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Abstract. Urea and guanidinium hydrochloride dissociate the 12 S protein of mustard and rapeseed to 1.8 S protein and the extent of dissociation depends on the concentration of the denaturant. Mustard (*Brassica juncea*) protein is more readily dissociated than the rapeseed (*Brassica campestris*) protein. The reagents denature the protein as evidenced by increase in viscosity, appearance of difference spectra and quenching of fluorescence. Rapeseed protein is denatured more readily than the mustard protein. Analysis of viscosity, spectral and fluoresence data suggests that the first event in the denaturation reaction is the perturbation of the aromatic amino acid residues followed by their exposure to the solvent medium and unfolding of the protein molecule.

Keywords. Mustard protein; rapeseed protein; denaturation; urea; guanidinium hydrochloride.

Introduction

Mustard and rapeseed proteins consist essentially of two fractions, a low and a high molecular weight protein. The high molecular weight protein, the 12 S protein, has been isolated to homogeneity (Gururaj Rao and Narasinga Rao, 1981). The protein from mustard (*Brassica juncea*) and rapeseed (*Brassica campestris*) differ in amino acid composition, subunit structure, susceptibility to proteolysis and secondary structure (Gururaj Rao and Narasinga Rao, 1981). In order to delineate further the differences between the two proteins the effect of denaturants, urea and guanidinium hydrochloride (GndHCl), has been studied. The results are reported in this communication.

Materials and methods

Mustard and rapeseed

Mustard seed (*B. juncea*) variety RT 590 and rapeseed (*B. campestris*) variety yellow sarson T 151 were obtained from Haryana Agricultural University, Hissar, India. Urea from British Drug House and guanidinium hydrochloride (GndHCl)

Abbreviations used: GndHCl, Guanidinium hydrochloride; RTIC, rotor temperature indicator and control; SDS, sodium dodecyl sulphate; S_{app}, apparent sedimentation coefficient; η_{red}, reduced viscosity; CD, circular dichroic; UV, ultra-violet.

from Sigma Chemical Co., St. Louis, Missouri, USA, were used. Urea was recrystallized once from 50% ethanol. All the other reagents were of analytical reagent quality.

Isolation of the 12 S protein

It was isolated by the procedure reported earlier (Gururaj Rao and Narasinga Rao, 1981).

Protein concentration

This was determined by measuring the absorbance of the solution at 280 nm. The values of $E_{1\,\mathrm{cm}}^{1\%}$ used were 9.9 for mustard protein and 10.8 for rapeseed protein (Gururaj Rao, 1980).

Ultracentrifugation

This was performed at room temperature (~27° C) in a Spinco Model E Analytical Ultracentrifuge equipped with rotor temperature indicator and control (RTIC) unit and phase plate schlieren optics using 1% protein solution in the appropriate solvent. The runs were made at 59,780 rpm. From the photographs taken at different intervals of centrifugation, apparent sedimentation coefficient, (S_{app}), was calculated. Enlarged tracings of the photographs were used to calculate the proportion of the protein components in the denaturing solvent.

Viscosity was measured at $30 \pm 0.1°$C using an Ostwald visco-meter having a flow time of 184 s with deionized water. Measurements were made with 1% protein solution at different denaturant concentrations.

Difference spectra produced by the addition of different concentrations of denaturant to the protein were recorded in the range 240–300 nm using a Perkin-Elmer Model 124 recording spectrophotometer. Protein solutions having an absorbance of 1.8 at 280 nm were used.

Fluorescence spectrum

The emission spectrum was measured in the range 300–400 nm after excitation at 280 nm. Protein solution having an absorbance of 0.05 at 280 nm was used. The blank for the measurement contained the appropriate concentration of the denaturant. Fluorescence measurements were made with Perkin-Elmer Model 203 fluorescence spectrophotometer at room temperature.

Unless otherwise measured, all the measurements were made in 0.025 M Tris-glycine buffer of pH 8.3.

Results and discussion

The value of the measured parameter in the presence of the denaturant was time-dependent. In urea solutions constancy of values was obtained at 48 h and in GndHCl solutions at 24 h. Hence the solutions were left at room temperature for 24 h in GndHCl and for 48 h in urea, and the measurement made.

The addition of urea to mustard 12 S protein dissociated it into 1.8 S protein; the extent of dissociation depended on urea concentration (figure 1). In 8 M urea solution the protein had completely dissociated into the 1.8 S component (figure 2A). GndHCl also dissociated the protein. The dissociating effect of GndHCl appeared to be greater than that of urea (figure 2A, B). For example at 2 M concentration, in urea the protein dissociated to the extent of 20% and in GndHCl it dissociated to 65%; at 4 M urea the dissociation was 80% and in 4 M GndHCl it was 100% (figure 2).

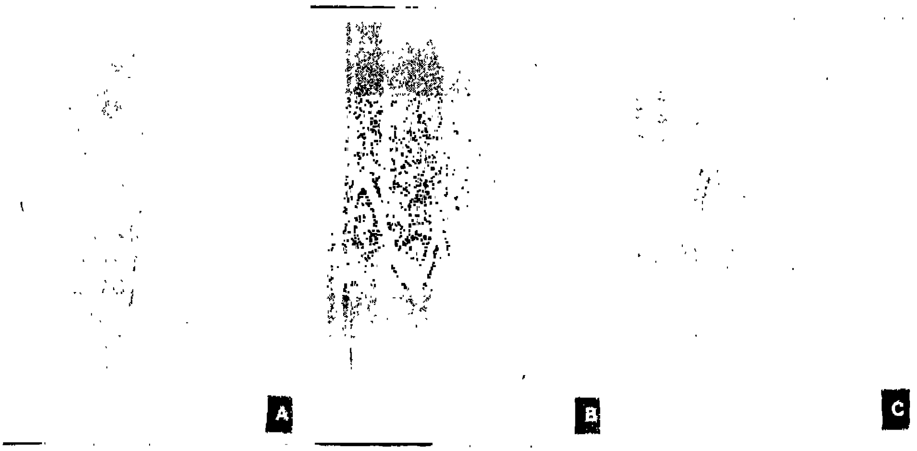

Figure 1. Sedimentation velocity pattern of mustard 12 S protein (in 0.025 M Tris-glycine buffer, pH 8.3).

A. Mustard 12 S protein; **B.** Mustard 12 S protein + 4 M urea; **C.** Mustard 12 S protein + 8 M urea.

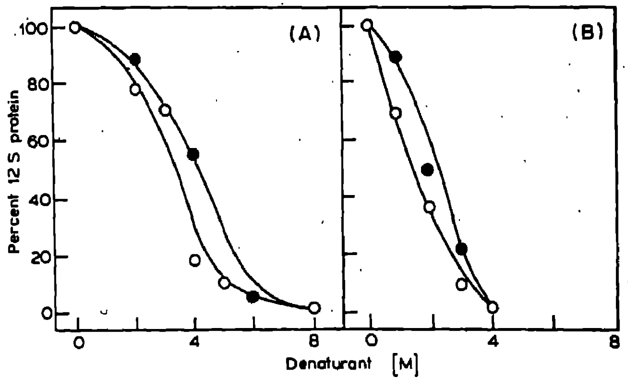

Figure 2. The per cent proportion of 12 S protein as a function of denaturant concentration.

A. In urea, (○), mustard protein; (●), rapeseed protein. **B.** In GndHCl, (○), mustard protein; (●), rapeseed protein.

In the case of rapeseed 12 S protein also urea or GndHCl caused dissociation of the protein to 1.8 S protein and the extent of dissociation increased with the denaturant concentration. However, either in urea or GndHCl rapeseed protein appeared to be dissociated to a lower extent than the mustard (figure 2A, B). In 4 M urea, the extent of dissociation of the mustard protein was 75–80% as against 55% with rapeseed protein (figure 2A). Similarly, in 2 M GndHCl, the dissociation of the mustard protein was 65% and that of the rapeseed protein 50% (figure 2B). Although much quantitative significance cannot be attached to the values, the trend appeared that the mustard protein dissociated more readily than the rapeseed protein.

Thus urea and GndHCl dissociated the 12 S protein of mustard/rapeseed directly to the 1.8 S protein without the formation of any intermediates. Sodium dodecyl sulphate (SDS) also dissociated the 12 S protein directly to the 1.8 S protein (Gururaj Rao and Narasinga Rao, 1980, 1983). This behaviour of the 12 S protein from *Brassica* species contrasts with that of other vegetable proteins. In the case of sunflower, 11 S protein SDS directly dissociates it to the 2 S protein, whereas urea or GndHCl dissociates it to an intermediate 7 S protien (Rahma and Narasinga Rao, 1981). The 11 S protein of guar seed also behaves similarly (Nath and Narasinga Rao, 1983). In the case of sesame α-globulin SDS and urea (and GndHCl) dissociate the 14 S protein to 2 S protein through intermediates, namely 7 S and 4 S proteins (Prakash and Nandi, 1975, 1977). This statement appears to be valid when the results obtained with 1% protein solution are compared. However, with a 3% protein solution, mustard and rapeseed 12 S protein also showed the presence of a 7 S protein at intermediate urea or GndHCl concentrations. This would suggest that the formation of the intermediate(s) is considerably less with mustard and rapeseed 12 S protein.

The reduced viscosity (η_{red}) of the mustard 12 S protein (measured at 1% protein concentration) had a value of 5.1 cc/g which is close to its intrinsic viscosity of 5.4 cc/g (Gururaj Rao and Narasinga Rao, 1981). Addition of urea increased η_{red} and at 8 M urea concentration it was 22 cc/g (figure 3). Even at this concentraton of urea, a plateau value was not obtained, although the observed value was close to

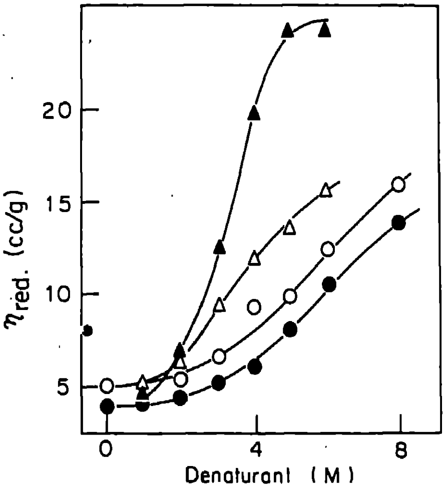

Figure 3. Reduced viscosity of the protein as a function of denaturant concentration (in 0.025 M Tris-glycine buffer, pH 8.3). (O), Mustard in urea; (●), rapeseed protein in urea; (Δ), mustard protein in GndHCl; (▲), rapeseed protein in GndHCl.

the value of 23 cc/g reported for proteins in random-coil conformation (Tanford, 1970). Addition of 2-mercaptoethanol did not alter the values significantly.

Rapeseed 12 S protein had a value of $\eta_{red} = 4.2$ cc/g close to its intrinsic viscosity of 4.5 cc/g (Gururaj Rao and Narasinga Rao, 1981). Addition of urea increased the value and at 8 M urea concentration the value was 14 cc/g (figure 3). This was much lower than that of the mustard 12 S protein at the same urea concentration; further even at other urea concentrations, the values were lower in the case of the rapeseed protein.

In GndHCl solution the mustard 12 S protein reached a value of 16 cc/g at a concentration of 6 M (figure 3) and no plateau value was reached. However, the rapeseed 12 S protein attained a constant value of 25 cc/g in the region of 4 to 6 M GndHCl concentration.

These results indicated that the rapeseed 12 S protein was more resistant to denaturation by urea; further with both the proteins a fully random-coil conformation was not obtained even in 8 M urea solutions. However, in GndHCl rapeseed protein was more readily denatured than mustard protein and at 4 to 6 M GndHCl concentration the protein attained a random-coil conformation. Mustard and rapeseed 12 S protein contain little or no α-helix; they consist of β-structure and random coil (Gururaj Rao and Narasinga Rao, 1981). However, in circular dichroic (CD) spectrum the mustard protein gives a value of -3940 deg cm^2/d for $[\theta]$ at 210 nm and the rapeseed protein a value of -4900 deg cm^2/d mol, suggesting that the latter has a more-ordered structure. The rapeseed 12 S protein was more resistant to hydrolysis by papain than the mustard protein. On the other hand chymotrypsin hydrolysed the rapeseed protein more readily than the mustard protein (Gururaj Rao and Narasinga Rao, 1981). Thus the responses of the two proteins to different treatments seemed to vary. The observed difference between the effect of urea and GndHCl on the mustard and rapeseed 12 S protein could be due to possible differences in their conformational stability.

The addition of urea to the mustard 12 S protein generated a difference spectrum in 260–300 nm region with troughs at 295, 287 and 278 nm (figure 4). The

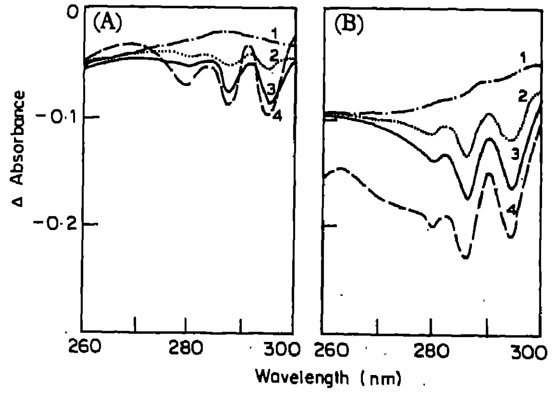

Figure 4. Differnce spectra (in 0.025 M Tris-glycine buffer, pH 8.3) recorded against protein blank.

A. (1) Mustard protein + 2 M urea; (2) mustard protein + 4 M urea; (3) mustard protein + 6 M urea; (4) mustard protein + 8 M urea.

B. (1) rapeseed protein + 2 M urea; (2) rapeseed protein + 4 M urea; (3) rapeseed protein + 6 M urea; (4) rapeseed protein + 8 M urea.

intensity of the troughs increased with urea concentration. Similar difference spectra were obtained with the rapeseed 12 S protein. However, the following differences were observed between the mustard and rapeseed protein: (i) in the case of the mustard protein the intensity of the troughs was less than with the rapeseed protein and (ii) intensity of the 294 nm trough was more than that of the 287 nm trough in the case of mustard 12 S protein and the converse was true in the case of rapeseed protein.

The minima at 278, 287 and 294 nm are due to the perturbation of tyrosine and tryptophan residues (Donovan, 1969). The mustard 12 S protein has 9 tryptophan residues and 16 tyrosine residues per 100,000 g and the rapeseed protein has 9 tryptophan and 14 tyrosine residues (Gururaj Rao and Narasinga Rao, 1981). Thus the increased intensity observed with the rapeseed protein cannot be correlated with its tyrosine and tryptophan content. Similarly in the case of the mustard 12 S protein, the greater intensity of the 294 nm peak than that of the 287 nm peak cannot also be correlated with the tyrosine and tryptophan content of the protein.

The blue shift observed can be ascribed to the binding of the denaturants to these chromophores or denaturation of the protein (Donovan, 1969). It is not clear which cause is responsible for the observed differences.

Similar difference spectra were generated by the addition of GndHCl. In this denaturant the intensities of the troughs were greater than in urea solution; the ΔA values at 287 nm are given in figure 5. Also the values with the rapeseed protein were higher than with the mustard protein.

The mustard and rapeseed 12 S protein give fluorescence emission spectrum with a maximum at 325 nm. The addition of the denaturant caused quenching of fluorescence; the extent of quenching increased with increase in denaturant concentration. Also at higher denaturant concentrations a red shift in the emission maximum occurred. Representative spectra obtained with the rapeseed 12 S protein in GndHCl solution are given in figure 6.

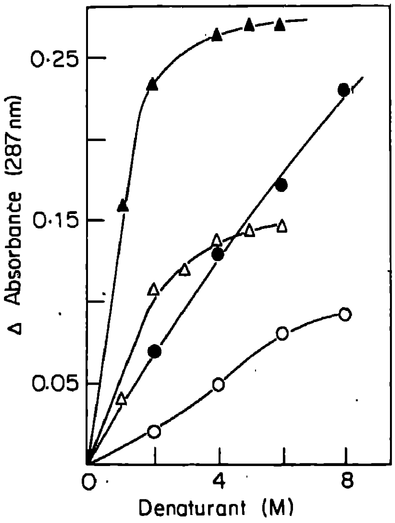

Figure 5. Value of absorbance change at 287 nm as a function of denaturant concentration: (○), mustard protein in urea; (●), rapeseed protein in urea; (Δ), mustard protein in GndHCl; (▲), rapeseed protein in GndHCl.

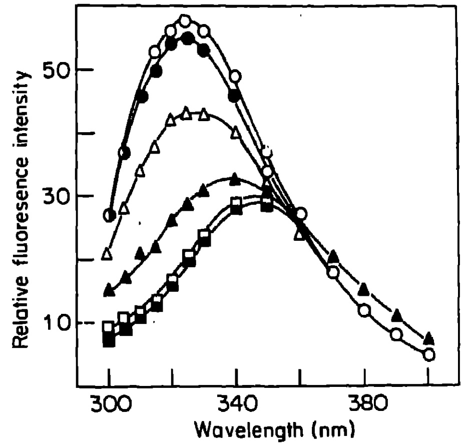

Figure 6. Fluorescence emission spectrum of rapeseed 12 S protein (in 0.025 M Tris-glycine buffer pH 8.3) in GndHCl solution; (○), protein; (●), protein + 1 M GndHCl; (△), protein + 2 M GndHCl; (▲), protein + 3 M GndHCl, (□), protein + 4 M GndHCl; (■), protein + 6 M GndHCl.

A plot of the ratio of the fluorescence intensity at 325 nm as a function of the denaturant concentration showed that the extent of quenching was greater in GndHCl than in urea, although the limiting value was the same in both the solvents (figure 7). Further with the same denaturant the extent of fluorescence quenching of mustard and rapeseed protein was the same, within the limits of experimental error.

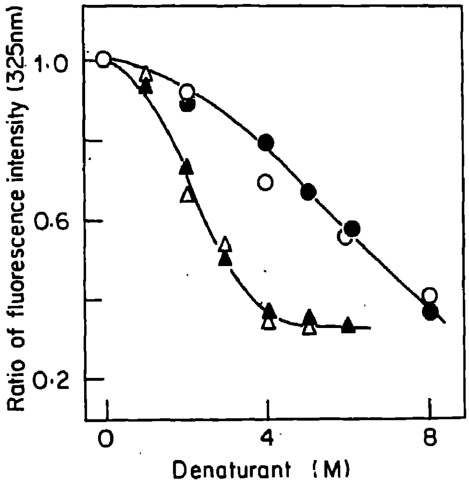

Figure 7. Ratio of fluorescence intensity at 325 nm as a function of denaturant concentration (in 0.025 M Tris-glycine buffer, pH 8.3): (○), mustard protein in urea; (●), rapeseed protein in urea; (△), mustard protein in GndHCl; (▲), rapeseed in GndHCl.

The sedimentation velocity experiments suggest that in either urea or GndHCl solution the mustard 12 S protein is more readily dissociated than the rapeseed protein, although it is not possible to attach quantitative significance to the observed differences. In viscosity measurements the rapeseed 12 S protein was less readily denatured by urea than the mustard protein and more readily by GndHCl. Fluorescence data did not indicate any difference between the rapeseed and mustard protein. On the other hand, the difference spectral data showed that rapeseed 12 S protein was more readily denatured by both urea and GndHCl than the mustard protein. Thus apparently conflicting conclusions would emerge from the three types of measurements.

Possibly the three techniques measured different facets of the conformational changes occurring due to the action of the denaturant. From the value of the measured parameter by any single technique as a function of denaturant concentration it is possible to calculate the fraction of the protein denatured as a function of denaturant concentration. If such values, obtained by diverse techniques fit the same curve it is usually taken as an indication that all the techniques are measuring the same conformational change (Tanford, 1970).

For such calculations, the value characteristic of the native protein and that of the fully denatured protein must be known. The value of the native protein can be obtained by measurement in the absence of the denaturant. The value of the denatured state is usually obtained from the plateau values at high concentrations of the denaturant. Reference to figures 3, 5 and 7 shows that plateau values were not always obtained. But the rapeseed 12 S protein in GndHCl solution gave plateau values in viscosity, difference spectra and fluorescence quenching measurements. Using these data, the fraction of protein denatured was calculated as a function of GndHCl concentration (Tanford, 1968).

The data of figure 8 show that the values obtained by different techniques did not fit the same curve; changes in ultra-violet (UV) spectra occur at lower GndHCl concentrations, followed by fluorescence and viscosity changes. The appearance of difference spectra is due to the perturbation of tryptophan and tyrosine residues; the 287 nm peak is due to tyrosine perturbation (Donovan, 1969). The

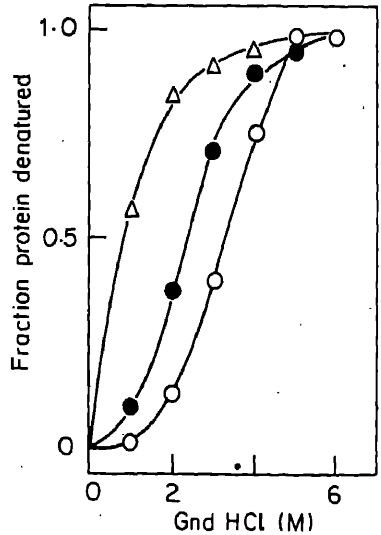

Figure 8. Fraction of rapeseed 12 S protein denatured as a function of GndHCl concentration: (O), η_{red} data; (Δ), A data; (●), fluorescence data.

325 nm fluorescence emission is due to the buried tryptophan residues (Teale, 1960); quenching of the fluorescence is due to the exposure of the buried tryptophan residues. Thus the first event in the denaturation reaction appears to be perturbation of the aromatic amino acid residues. This is followed by the exposure of the buried aromatic amino acids and finally by a conformational change leading to an increase in hydrodynamic volume.

Such an analysis of the observed data is, however, complicated by the dissociation of the oligomeric protein by the denaturant. If dissociation and denaturation of an oligomeric protein can be considered as equivalent phenomena (Lapanje, 1978) then the above analysis would have validity.

This analysis does not still answer the question why the two proteins which show only minor differences in amino acid composition and secondary structure, behave differently towards the denaturants. Urea and GndHCl act as bifunctional reagents and proteins bind one molecule of denaturant per two peptide residues and through aromatic amino acid residues (Robinson and Jencks 1965; Nozaki and Tanford, 1970; Prakash *et al*, 1981). Denaturation of proteins by these reagents occurs by breaking of hydrogen and hydrophobic bonds. The hydrophobicity and fractional charge units per residue of the mustard and rapeseed 12 S protein was calculated by the procedure outlined by Bigelow (1967) using data of amino acid composition of the two proteins (Gururaj Rao and Narasinga Rao, 1981). The hydrophobicity value was 808 cals/residue for mustard protein and 721 cals/residue for rapeseed protein. Similarly' the fractional charge per residue was 0.375 charge unit for mustard protein, and 0.386 charge unit for rapeseed protein. These differences are not significant and may not explain the differing behaviour of the two proteins towards urea and GndHCl. Prakash *et al.* (1981) did not find any correlation between protein hydrophobicity and the number of denaturant molecules bound per protein molecule.

The subunit composition of the two proteins as determined by SDS-polyacrylamide gel electrophoresis, shows significant differences (Gururaj Rao and Narasinga Rao, 1981). The amino acid compostion of the subunits and their geometry in the oligomeric protein may play a role in the denaturation of these proteins.

Acknowledgement

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Effect of calcium on synthesis of dipicolinic acid in *Penicillium citreoviride* and its feedback resistant mutant

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Abstract. Dipicolinic acid synthesis in *Penicillium citreoviride* strain 3114 was inhibited by Ca^{2+} ions, but not by Ba^{2+}, Cu^{2+} or Fe^{2+}. Among the metals tested, only Zn^{2+} inhibited the synthesis of dipicolinic acid and promoted sporulation. None of these metals reversed the inhibition by Ca^{2+} or Zn^{2+}. A mutant 27133-*dpa-ca* selected for resistance to feedback inhibition by dipicolinic acid: Ca^{2+} complex showed cross-resistance to inhibition by dipicolinic acid: Zn^{2+}. Both 3114 and 27133-*dpa-ca* excreted a number of aliphatic and amino acids during secondary metabolism of dipicolinic acid. In the presence of 1000 ppm of Ca^{2+}, accumulation of citric acid and α-aminoadipic acid was completely inhibited under conditions of inhibition of dipicolinic acid in parent strain 3114 but not in the mutant. Citric acid with or without Ca^{2+} did not inhibit the *de novo* synthesis of dipicolinic acid in the strain 3114. In fact, citric acid in the presence of Ca^{2+} improved significantly rate of dipicolinic acid synthesis. Apart from resistance to feed back inhibition by dipicolinic acid: Ca^{2+} complex, mutant differed from the parent in three other aspects *viz.* (i) dipicolinic acid synthesis was not subject to catabolite repression by glucose, (ii) sporulation as well as dipicolinic acid synthesis was dependent on the presence of Ca^{2+} ions in the medium and (iii) Mg^{2+} requirement for the mutant increased three fold. Higher requirement of the Mg^{2+} could be partially relieved by Ca^{2+} during secondary metabolism. The results support the inference that *de novo* synthesis of dipicolinic acid is regulated through feedback inhibition by dipicolinic acid: Ca^{2+} complex.

Keywords. *Penicillium citreoviride*; dipicolinic acid; secondary metabolite; DPA regulation; feedback inhibition.

Introduction

Previous studies from this laboratory, have shown that (i) dipicolinic acid (pyridine, 2,6-dicarboxylic acid; DPA) was synthesised by the mold *Penicillium citreoviride* as a secondary metabolite, (ii) its *de novo* synthesis was inhibited by calcium, and (iii) though the mold showed increased sporulation in the presence of calcium, no detectable amount of DPA could be observed in the spores (Kalle 1978; Kalle and Khandekar 1983). This was in contrast to the observations in bacteria where DPA and calcium were incorporated directly into the spore cortex (Forman and Aronson, 1972; Tamir and Gilvarg, 1974). It was proposed that inhibition of DPA synthesis in the mold was as a result of feedback inhibition by DPA : Ca complex and that the increased sporulation in the presence of calcium was not related to the DPA metabolism (Kalle and Khandekar, 1983). Implicit in this mechanism, was an assumption that action of calcium in inhibiting DPA synthesis was specific

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Abbreviations used: DPA, Dipicolinic acid—2,6-dicarboxylic acid; NTG, nitrosoguanidine.

and not related directly to sporulation. In this communication, we present evidence to show that among the metals which complex with DPA, only Ca^{2+} and Zn^{2+} exhibited the ability to inhibit DPA synthesis. Furthermore, mutants selected for resistance to feedback inhibition by DPA : Ca complex also lost their sensitivity to DPA : Zn complex. Such a mutant continued to show increased sporulation and produce DPA in the presence of calcium.

Materials and methods

Growth and studies

Media, maintenance of the mold *P. citreoviride* strain NRRL 3117, growth conditions and estimation procedures were described in our earlier communication (Kalle and Khandekar, 1983). Cultures were grown in 100 ml of synthetic medium (pH 7.0) in the presence and absence of Ca^{2+} ($CaCl_2$), Mg^{2+} ($MgCl_2$), Zn^{2+} ($ZnCl_2$), Fe^{2+} ($FeCl_2$), Cu^{2+} ($CuSO_4$) and Ba^{2+} ($BaCl_2$). All experiments were carried out at 30° C on a rotary shaker in 500 ml flasks. Accumulated products were analysed by paper chromatography with a solvent system comprising of butanol : acetic acid : water in the ratio of 12 : 3 : 5 v/v. Amino acids were reacted with ninhydrin and compared visually with standards. Aliphatic acids were visualised by spraying with aniline-xylose mixture followed by heating at 105° C for 10 min (Schwartz, 1963). The presence of citric acid was also confirmed by standard pentabromoacetate method (McArdle, 1965). The effect of citrate on DPA synthesis was checked by adding pure citric acid to the medium in the presence and absence of added Ca^{2+} to the medium.

Isolation of mutants resistant to feedback inhibition by DPA : Ca complex

Strain 3114 was grown on Sabouraud's agar medium for 10–14 days till sporulation was completed. Spores were suspended to a concentration of 10^6 per ml in 0.05 M Tris-maleate NaOH buffer (pH 9.0) containing 3 mg/ml of nitrosoguanidine (NTG) and incubated for 30 min at 30° C. Action of NTG was terminated by dilution and plating directly on several Sabouraud's agar plates. The plates were incubated for 3-4 days at 30° C and colonies were transferred by replica plated to two agar plates containing synthetic medium supplemented with 1000 ppm Ca^{2+}, added as $CaCl_2$. On one set of these plates 3 ml of 0.1% ferrous sulphate solution was gently and uniformly poured without disturbing the colonies. Within 5 min, DPA producing colonies turned pink, because of the formation of a coloured DPA : Fe complex. Colonies from the other plate were marked, purified further and checked to determine if they produced DPA when grown in the presence of 1000 ppm Ca^{2+} in the synthetic medium. The DPA produced by the mutant was purified by ion-exchange chromatography (Kalle and Khandekar, 1983) and its identity confirmed spectrophotometrically by comparison with standard DPA and also that obtained from the parent culture. The mutant strain 27133-*dpa-ca* was chosen for further studies. It was routinely maintained on Sabouraud's agar supplemented with 1000 ppm of Ca^{2+}.

Results and discussion

Effect of divalent metals on DPA synthesis in P. citreoviride strain 3114

Among the metal ions tested only Ba^{2+} showed a partial reversal of inhibitory effects by Ca^{2+} of DPA synthesis, whereas Cu^{2+}, Zn^{2+} and Fe^{2+} did not show similar effects (figure 1). Cu^{2+} when added alone (*i.e.* in absence of Ca^{2+}) showed stimulation of DPA synthesis and final levels reached in the medium were significantly higher than the control. The most interesting effect observed was inhibition of DPA synthesis by another metal ion Zn^{2+}, which emulated all the effects of Ca^{2+} including stimulation of sporulation in this mold. All the metal ions tested here (Ba^{2+}, Cu^{2+}, Zn^{2+}) have been shown to have a capacity to replace Ca^{2+} ions from DPA : Ca complex in bacterial spores (Gould and Dring 1974). Influence of metal ions on growth in relation to their effect on DPA synthesis is shown in the data presented in table 1. Generally, the addition of metal ions at 100 ppm did not affect adversely the growth pattern of the mold. Furthermore, on solid media, sporulation was extensive when Ca^{2+} or Zn^{2+} was present either alone or in combination with other metals. It is interesting to note that the final pH of the medium was alkaline whenever there was extensive sporulation. This has also been observed during sporulation in bacteria. In the presence of Ca^{2+} or Zn^{2+} there was a net inhibition of *de novo* DPA synthesis as indicated by low yields of this metabolite per unit increase in cell mass, and there was no net synergistic effect even under conditions when both Ca^{2+} and Zn^{2+} were present.

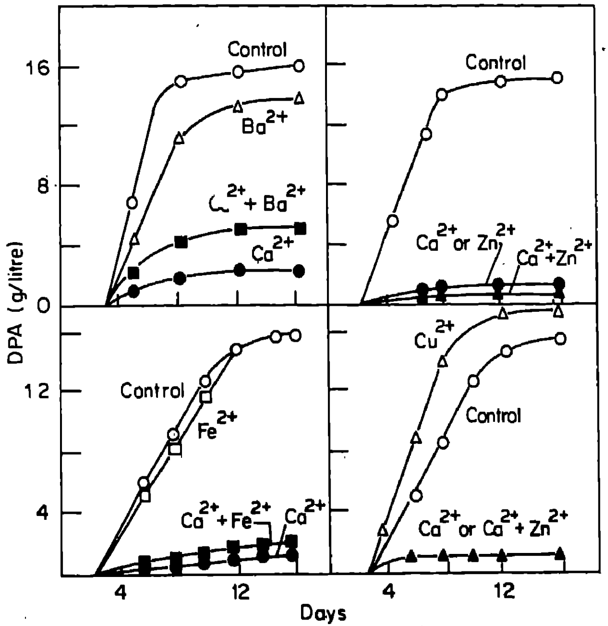

Figure 1. Influence of metal ions on DPA synthesis in *P. citreoviride* concentration of metal ions was 100 ppm each. All the flasks including the control contained 0.1% of $MgSO_4 \cdot 7H_2O$.

Table 1. Effect of metal ions on net synthesis of DPA in *Penicillium citreoviria.*

Metal ions	Final pH	DPA g/litre	DPA g/mg DW cells	Growth mg DW cells per 100 ml medium
Nil	3.8	14.0	0.27	5.4
Ca^{2+}	8.3	1.2	0.02	6.1
Zn^{2+}	8.0	0.9	0.02	5.9
Ba^{2+}	8.0	13.0	0.22	5.8
Cu^{2+}	5.2	17.1	0.36	4.9
Fe^{2+}	5.7	15.0	0.23	5.9
$Ca^{2+}+Zn^{2+}$	8.2	0.5	0.01	6.2
$Ca^{2+}+Ba^{2+}$	8.2	4.5	0.06	6.0
$Ca^{2+}+Cu^{2+}$	7.6	0.5	0.01	4.8
$Ca^{2+}+Fe^{2+}$	8.4	0.9	0.012	7.1

Concentration of all metal ions was maintained at 100 ppm. In flasks containing two metal ions, each was added at 100 ppm level. DPA and total mycellial concentrations was measured after 10 days of incubation at 30° C on rotary shaker (100 ml medium/ 500 ml flask).

Influence of calcium on accumulated products

Calcium has been shown to have an important role to play in the transport of aliphatic acids and amino acids in some molds (Ring *et al*, 1970; Hackette *et al*, 1970; Cameron and Le John, 1972) and even in bacteria prior to events leading to sporulation (Murrell, 1967). It is possible to consider a role for calcium in which it aids the organism to retain the necessary pool of certain metabolites required for sporulation. In bacteria, there is evidence that some of the organic acids (tricarboxylic acid — cycle intermediates) accumulate prior to sporulation and these are utilised rapidly when sporulation is triggered, with the result that pH shifts rapidly to the alkaline side (Murrell, 1967). Somewhat similar conditions were observed in experiments where Ca^{2+} stimulated sporulation and inhibited DPA synthesis.

Examination of supernatant fluids from the culture in the presence and absence of Ca^{2+} showed that both qualitatively and quantitatively less number (and amount) of acids accumulated in the presence of calcium (figures 2 and 3). Only α-amino adipic acid and citric acid were identified using standards, since they have been reported to be key intermediates in biosynthesis of DPA (Tannenbaum and Kaneko 1964); other amino acids were not identified. Accumulation of both these intermediates was completely inhibited in the presence of Ca^{2+} under conditions which inhibited DPA synthesis. This inhibition was not observed in the mutant 27133-*dpa-ca* which was selected for resistance to feedback inhibition by DPA : Ca complex. In order to determine if accumulation of intermediates, such as amino acids, aliphatic acids, etc. showed any correlation with pH changes and sporulation, they were monitored periodically during growth by chromatographic analysis. Relative intensities of these products were recorded (figure 3). In the absence of Ca^{2+}, both aliphatic acids and amino acids paralleled DPA synthesis and

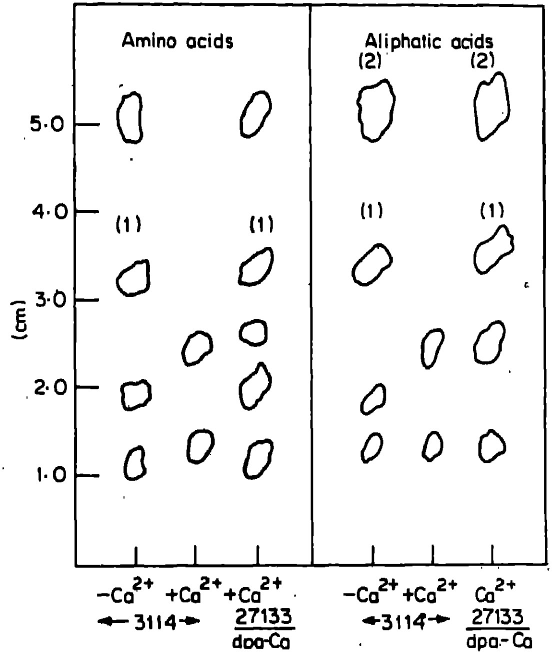

Figure 2. Chromatographic pattern of the accumulated products in growth medium of strain 3114 and 27133-*dpa-ca*. Strains were grown in the presence and absence of 1000 ppm of Ca^{2+} for 10 days at 30°C on rotary shaker. The culture fluid after separation of mycellia was directly spotted on paper (1) α-aminoadipic acid; (2) citric acid.

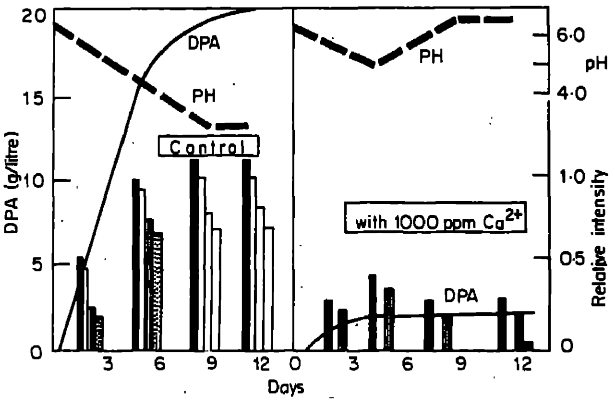

Figure 3. Accumulation of aliphatic acids and amino acids during various stages of DPA synthesis. Strain 3114 was grown in the presence of 1000 ppm Ca^{2+} and culture fluid was chromatogramed. The results are plotted as relative intensities on a 0–1 scale.

Alimphatic acids ■ ; Citric acid ☐

Amino acids ▥ α-Aminoadipic acid ▨

decrease in pH. In the presence of Ca^{2+}, citric acid and α-aminoadipic acid were absent in the culture fluid throughout the growth of the organism. Other acids accumulated intially and then decreased as a function of time. The pH changes paralleled the accumulation pattern in that, it decreased initially and then increased till sporulation was complete. Zinc mimicked all the effects of calcium both in the parent strain 3114 and mutant 27133-*dpa-ca*.

Influence of citric acid on DPA synthesis

According to the pathway proposed by Tanenbaum and Kaneko (1964), citric acid is a precursor to DPA in *P. citreoviride* (see figure 8). Evidence available so far has indicated that Ca^{2+} tends to prevent accumulation of organic acids into the medium. Does it help in retention of these acids inside the cell, thus making them available for growth and sporulation ? Alternatively, does it have any role in transport of these acids into the cells ? We have attempted to answer these questions indirectly by studying the ability of the strain to use citrate in the presence of normal available carbon source-sucrose. Data are presented in figure 4. Addition of citrate or citrate with calcium had no inhibitory effect on *de novo* synthesis. In other words, citrate effectively reversed inhibitory effects of Ca^{2+} on DPA synthesis. In fact, there was a significant increase in the rate of DPA synthesis in the presence of citrate, an observation which may be explained if this metabolite was a precursor of DPA in this mold. The fact that citrate competes with sucrose in the presence of calcium is shown indirectly from the pattern observed in utilization of sucrose. The rate of sugar taken up during the DPA synthesis in the presence of calcium alone,

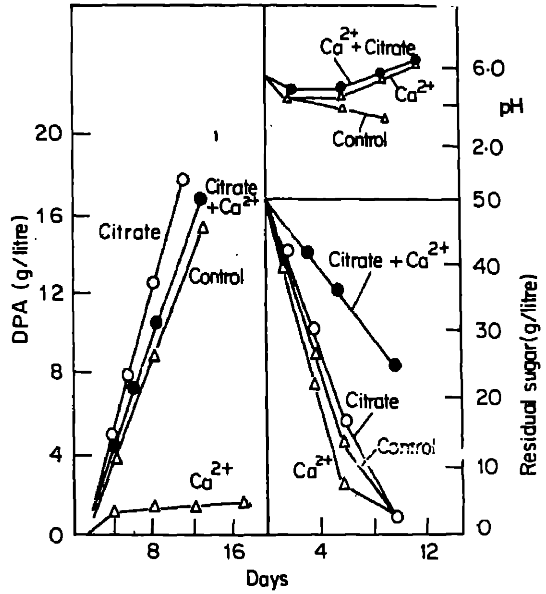

Figure 4. Influence of citric acid on DPA synthesis in the presence and absence of 1000 ppm Ca^{2+}. In the absence of Ca^{2+}, citric acid accumulation upto 3 g/litre. In the presence of Ca^{2+}, all of the citrate was used up from the medium. Concentration of citrate in the medium 10 g/litre.

sucrose and sucrose with citrate was more or less the same. However, in the presence of calcium and citrate, sugar uptake was greatly reduced. Since growth rates in all the cases were the same, we feel that citrate competes with sucrose more efficiently in the presence of calcium as a carbon source, and also possibly, as a precursor to DPA synthesis.

Properties of mutant 27133-dpa-ca

The mutant 27133-*dpa-ca* differed from its parent 3114 in several properties, particularly in its response to calcium. The mutant produced DPA in the presence of 1000 ppm Ca^{2+}, but the rate of DPA synthesis and the sugar uptake were calcium dependent (figure 5). In the presence of calcium, the amount and the rates of DPA synthesis were twice than that observed in the absence of calcium. In fact, there was a rapid drop in pH which had to be neutralised to enable further synthesis of DPA. Another distinguishing property of the mutant, was that unlike its parent (Kalle and Khandekar, 1983), the DPA synthesis was not subject to catabolite repression. In all these experiments, starting sugar was 15–20% at which DPA synthesis was repressed in the parent strain. Furthermore, the mutant unlike its parent sporulated only when Ca^{2+} was present in the medium. It did not sporulate in the absence of Ca^{2+}, a property not observed with the parent strain. The mutant showed resistance to inhibition by Zn^{2+} of *de novo* DPA synthesis, suggesting a common site of action for the two metal ions.

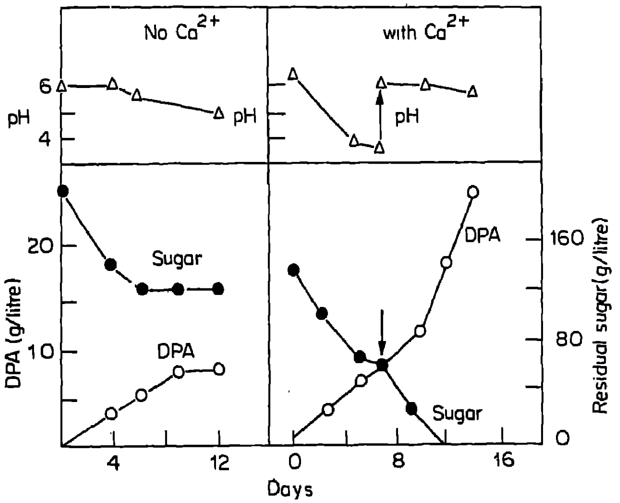

Figure 5. Effect of Ca^{2+} on DPA synthesis in the mutant. *P. citreoviride* 27133-*ca-r-*. Medium contained 1000 ppm Ca^{2+} added as $CaCl_2$. In the presence of Ca^{2+}, the pH was adjusted to 6.0 with 1 N NaOH on 8th day of fermentation.

Requirement for Mg^{2+} for DPA synthesis

One of the possibilities for limitation in sugar uptake and DPA synthesis in the absence of calcium in the mutant 27133-*dpa-ca* could have been due to the increased requirement of Mg^{2+} for the mutant. A consequence of this assumption would be that calcium had a sparing action on the Mg^{2+} requirement of the strain resulting in better rates of sugar uptake and higher DPA yields. In the case of the parent strain 3114, there is an obligate requirement of $MgSO_4$ for synthesis of DPA and at concentrations less than 0.05% of $MgSO_4$, the DPA levels in the medium were drastically reduced (figure 6). In the mutant strain, however in the presence of Mg^{2+} (0.1% $MgSO_4$), the sugar uptake and DPA synthesis came to a halt after 6 days. It resumed only when additional Mg^{2+} (0.05% $MgSO_4$) was added (figure 7). In the presence of 1000 ppm of Ca^{2+}, there was no cessation of DPA synthesis and in fact addition of 0.05% $MgSO_4$ showed significant improvement in DPA synthesis. When initial concentration of $MgSO_4$ was reduced to 0.05%, which was considered optimal for the parent strain, the rate of DPA synthesis was drastically reduced but improved after further supplementation of

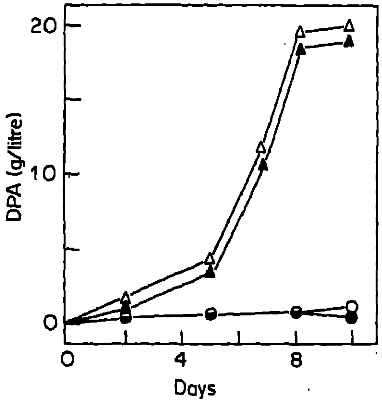

Figure 6. Effect of Mg^{2+} concentration on DPA synthesis in strain 3114. The mold was grown in synthetic medium with 0.1% $MgSO_4 \cdot 7H_2O$ for 6 days at 30° C. Mycellia centrifuged, washed and starved for one hour in medium without Mg^{2+}. It was then transferred to the medium containing at following concentrations of $MgSO_4$.
(●), 0%; (○), 0.025%; (▲), 0.05% and (△), 0.1%.

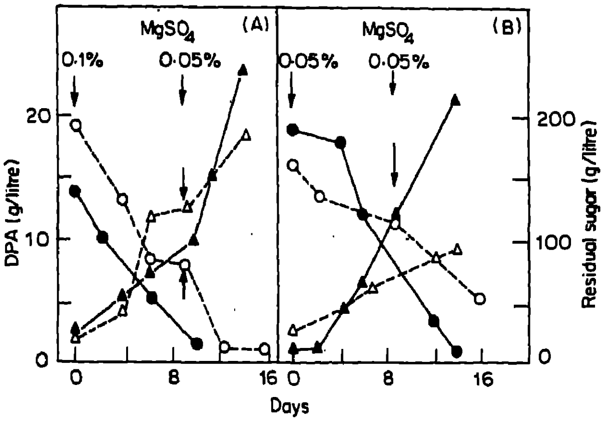

Figure 7. Effect of Ca^{2+} on magnesium requirement of the mutant 27133-*dpa-ca*.
DPA: (△), (No Ca^{2+}); (▲), (Ca^{2+}: 1000 ppm);
Sugar: (●), (No Ca^{2+}); (○), (Ca^{2+}: 1000 ppm).

Mg^{2+} ions. In the presence of calcium, this level of $MgSO_4$ was more than adequate for continued DPA synthesis (figure 7). This type of sparing action was not observed in the parent strain which was inhibited even at 10 ppm Ca^{2+} (Kalle and Khandekar, 1983).

Role of calcium in regulating biosynthesis of secondary metabolite DPA

Importance of trace metals in the regulation of secondary metabolite biosynthesis has been reviewed in great detail by Weinberg (1970). The metal ions may either inhibit or stimulate secondary metabolite synthesis and there is often a linear correlation between the yield of the product and the log of concentration of the metal ion. The mechanism of action of so called "key" metals has not been properly understood, and it is believed that they may influence the secondary metabolite biosynthesis by interferring at pre-transcription, transcription, as well as at post-translation level (Weinberg, 1970). We had proposed earlier that action of Ca^{2+} ions in inhibiting DPA synthesis was due to the feedback inhibition by DPA : Ca complex (Kalle and Khandekar, 1983). The data presented here substantiates this hypothesis on the basis of the following evidence.

Specificity of Ca^{2+}: All the metal ions tested here have capacity to chelate with DPA but their stability constants (log *K*) vary significantly anywhere from 10 for Cu^{2+} to 2.4 for Mg^{2+} (Murrel, 1967). Three of the metal ions Cu^{2+}, Zn^{2+} and Fe^{2+} had chelate stability for DPA greater than that of Ca^{2+}, and Ba^{2+} and Mg^{2+} had lower stability. And yet only Ca^{2+} and Zn^{2+} inhibited *de novo* DPA synthesis.

Resistance to feedback inhibition by DPA : Ca complex: The DPA synthesis in the mutant 27133-*dpa-ca* selected for resistance to feedback inhibition by DPA : Ca complex, was not inhibited by Zn^{2+} suggesting that the site of action for DPA synthesis for Ca^{2+} and Zn^{2+} were probably the same in this mold.

Inhibition of precursors of DPA by DPA : Ca complex: In the presence of Ca^{2+} and under conditions of DPA inhibition two of the metabolites, citric acid and α-aminoadipic acid were inhibited completely and selectively. Accumulation of other organic acids was inhibited only partially in the presence of Ca^{2+}. According to the pathway proposed by Tanenbaum and Kaneko (1964) both these metabolites are probably precursors of DPA biosynthesis (figure 8). Accumulation of α-aminoadipate can be explained if it is derived from α-ketoadipic acid which is not used for DPA synthesis.

Accumulation of citrate and its disappearance from the medium in the presence of calicum can be explained only if we consider citric acid as one of the precursors of DPA synthesis. Here calcium may be assisting in better citrate utilisation by the mold as evidenced from the fact that it successfully competes with sugar in supporting growth and DPA synthesis. It did not inhibit the *de novo* synthesis of DPA suggesting that the controlling site for DPA metabolism is probably between citrate and α-ketoadipic acid in the pathway. It is interesting to note that α-ketoadipic acid is a common precursor both to DPA and lysine biosynthesis and in

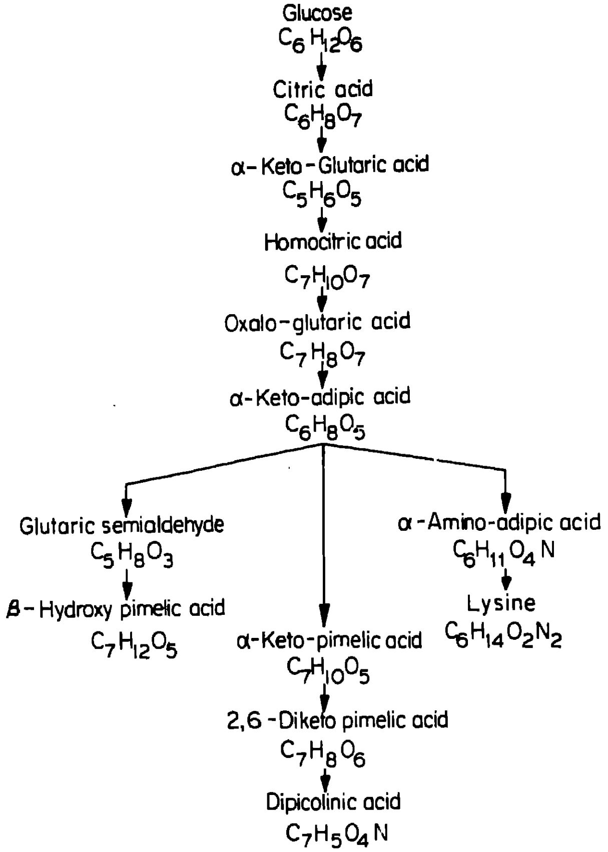

Figure 8. Proposed pathway for DPA synthesis in *P. citreoviride* adapted from Tanenbaum and Kaneko (1964).

P. chrysogenum, enzyme homocitrate dehydrogenase is the controlling enzyme specifically inhibited by lysine (Demain, 1966). It is likely that the same enzyme may also be inhibited by DPA : Ca complex.

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Interaction of lysozyme with antibiotics — binding of penicillins to lysozyme

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Abstract. Binding of lysozyme with the antibiotics such as penicillin-G, penicillin-V and methicillin at different concentrations and pH was studied by equilibrium dialysis. Co-operative binding isotherms were observed at pH 5.0, 7.0 and 9.0 with all the penicillins and the binding ratios decreased slightly with the increase of pH. The Gibbs free energy change calculated on the basis of Wyman's binding potential concept decreased slightly with the increase of pH indicating slight decrease in the binding strength at higher pH in the case of all penicillins.

The ultra-violet difference spectra of lysozyme-penicillin complexes showed a less intense peak in the region of 284-300 nm at pH 5.0. Only penicillin-G complex had a peak at pH 7.0 at these wavelengths with less intensity compared to that at pH 5.0. However, none of the penicillins showed discrete peaks in this region at pH 9.0. The appearance of peaks in the difference spectra of all these complexes at pH 5.0 and with only penicllin-G complex at pH 7.0 in the aromatic region indicated hydrophobic interactions with tryptophan residues as the binding sites. In addition, the ionic interactions with lysine residues in lysozyme were also occurring.

The conformational changes induced by the binding of penicillins to lysozyme monitored by circular dichroism showed a slight decrease in the aromatic bands in the 320-250 nm region. However, in the 250-200 nm region, $[\theta]_{222\text{nm}}$ values obtained at various concentrations of penicillins in the complex indicated an increased α- helical content generating a more ordered structure. These results led to the conclusion that both the hydrophobic and electrostatic interactions prevail in the binding of penicillins to lysozyme.

Keywords. Lysozyme; penicillins; co-operative binding; helical content.

Introduction

A study of the binding of penicillins to proteins is important in the understanding of its function in absorption, transport, anti-bacterial activity, toxicity etc. The reversible binding (Klotz *et al.*, 1950; Rolinson and Sutherland, 1965) of penicillins and other drugs to proteins demonstrated using ultrafiltration (Keen, 1965, 1966; Bird and Marshall, 1967) *in vitro* and *in vivo* has been a subject of extensive discussion on their probable significance. However, the exact nature of the chemical interactions and the binding sites involved were not clearly identified. Ionic binding of penicillins to lysozyme has been studied in detail by pulse radiolysis (Philips *et al.*, 1970, 1973), proton magnetic resonance (Mitsumori *et al.*, 1980), fluorimetric and ultra-violet (UV) difference spectroscopy (Felsenfeld and

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Abbreviations used: CD, Circular dichroism; UV, ultra-violet.

Handschumacher, 1967), x-ray diffraction technique (Johnson, 1967), radio isotope labelling (Corran and Waley, 1975) and equilibrium dialysis (Klotz *et al.*, 1950). These studies reveal that the side chains of penicillins are involved in the hydrophobic interactions especially with tryptophans in the protein, while the carboxylate anionic groups in penicillins bind at the cationic sites such as lysines. The stoichiometry reported for lysozyme-penicillin complexes from these studies is found to vary with the type of penicillin which basically differs in the hydrophobic character of R group as well as the nature and number of cationic sites available for binding through electrostatic interactions.

In the present investigation lysozyme was chosen as a model protein because its three dimensional structure, its physico-chemical characteristics and its biological function were extensively studied (Johnson and Philips, 1965; Rupley, 1967; Rupley and Gates, 1967; Sophianopoulos and Van Holde, 1964; Sophianopoulos and Weiss, 1964; Blake *et al.*, 1965, 1967; Philips, 1966). The interaction of lysozyme with penicillins such as penicillin-G (benzyl penicillin), penicillin-V (Phenoxy methyl penicillin), and methicillin (2,6-dimethoxy phenyl penicillin) at various concentrations and pH, using equilibrium dialysis technique are described here. The thermodynamic parameters of binding were evaluated. The nature of the binding sites and the interactions there at was determined by UV difference spectra, and the conformational changes accompanying the binding were followed by circular dichroism (CD) technique. However, the enzymatic activity of lysozyme due to penicillin binding was not studied.

Materials and methods

Hen egg white lysozyme (3X crystallized, lot No. 6876), penicillin-G, penicillin-V and methicillin obtained from Sigma Chemical Company, St. Louis, Missouri, USA were used. Imidazole (British Drug House, Poole, England) was recrystallized thrice from benzene to remove UV absorbing impurities. All other reagents used were of analytical reagent grade and the solutions were prepared using double distilled water. For equilibrium dialysis, membrane tubing from Union Carbide Corporation, Chicago, Illinois, USA, was used. UV-Visible spectrophotometer (Carl Zeiss) was used to measure the concentration of lysozyme and penicillins. $E^{1\%}_{280\ \mathrm{nm}} = 26.4$ for lysozyme (Sophianopoulos *et al.*, 1962) was used to calculate the concentration of lysozyme.

The concentrations of penicillins are determined by absorbance measurements at 325–335 nm of penicillinic acid mercuric mercaptide of the respective penicillins formed in quantitative yield on heating at 60° C with 1.2 M imidazole and 1 mM mercuric chloride solution as described elsewhere (Bundgaard and Ilver, 1972).

Jasco J-20 spectropolarimeter previously calibrated with (+)-10 camphor sulphonic acid (De Tar, 1969) was used to record the CD spectra. Beckmann spectrophotometer Model-25 was used to record the UV difference spectra.

In the equilibrium dialysis experiments, 5 ml of 0.2% lysozyme (0.14 mM) was dialysed against 10 ml of the penicillin solution at various concentrations in the same buffer medium as described earlier (Subramanian and Venkatappa, 1982). The time required for equilibration was found to be o h at 25 °C. The binding ratios '*r*', the number of mol of penicillin bound per mol of the protein at various free penicillin concentrations [D], are calculated in a way similar to that of Rosenberg and Klotz (1961).

The buffers used were; 0.01 M sodium acetate–acetic acid buffer, pH 5.0, 0.01 M Na_2HPO_4-KH_2PO_2 buffer, pH 7.0 and 0.01 M glycine buffer, pH 9.0. The binding studies were carried out using 0.14 mM lysozyme and varying the penicillin concentration (1 – 7 mM), while the UV difference spectra was recorded at 0.07 mM enzyme concentration.

The CD spectra of lysozyme at 23.5 and 35 μM concentrations and its complex with penicillin were measured at 25° C in a 0.5 mm cell in the 250-200 nm region and using a 10 mm cell in the 320-250 nm region respectively. The results are represented as molar residue ellipticity, $[\theta]_\lambda$ in deg cm^2 $dmol^{-1}$, calculated in a manner similar to that of Adler *et al.* (1973) taking a value of 111 as mean residue weight for lysozyme (Warren and Gordon, 1970).

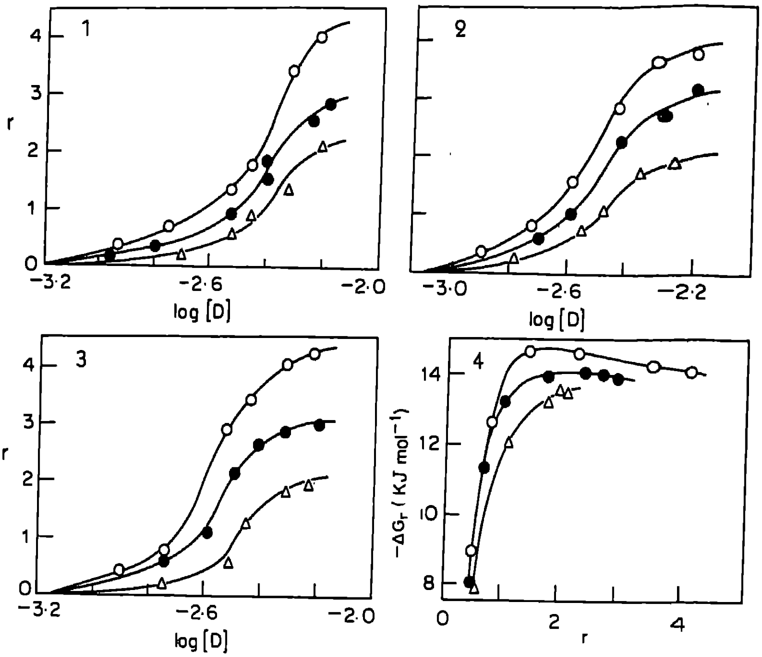
Results and discussion

Binding isotherms obtained for the interaction of lysozyme with penicillin-G, penicillin-V and methicillin showed co-operative binding at pH 5.0, 7.0 and 9.0. The binding isotherms of *r vs.* log [D] for these penicillins are shown in figures 1, 2 and 3 respectively.

Wyman's binding potential concept was used to interpret these binding isotherms as described by Jones and Manley (1979). The apparent Gibbs free energy of binding, ΔG_a was calculted from *r vs.* log [D] plot from which the apparent free energy of binding per bound ion was calculated using the relation $\Delta G_r - \Delta G_a / r$. The results of the interaction studies with penicillin-G at various pH is shown in figure 4 as a typical example from which the maximum values of ΔG_r are computed. In a similar manner, the ΔG_r values were calculated for penicillin-V and methicillin interactions.

The binding of penicillins with lysozyme decreased slightly with the increase of pH. This was due to the decrease in the net positive charge on the protein with the increase of pH. Accordingly the number of available binding sites on lysozyme decreased from 4 at pH 5.0 to 3 at pH 7.0 and then to 2 at pH 9.0 for all the penicillins. The number of binding sites, the values of ΔG_r and binding constant K at various pH for the interacton of lysozyme with penicillins are given in table 1. It was observed that the magnitude of ΔG_r decreased only slightly with the increase of pH for all penicillins indicating a slight decrease in the strength of binding at higher pH.

The UV difference spectra of lysozyme-penicillin complexes at pH 5.0 showed peaks of low intensity around 284-286 nm in the case of penicillin-G complex, around 296–297 nm in penicillin-V complex and at 300 nm in the case of methicillin complex, thus showing a bathochromic shift in the peaks with the variation in the nature of the side chain group of penicillin nucleus. The intensity of the peaks, however, increased with the increase in the concentration of penicillin in the complex. Figure 5 shows the UV difference spectra of lysozyme-penicillin-G complex taking it as a typical example. None of the penicillin complexes showed any peaks in the 284–300 nm region at pH 7.0 and 9.0 except in the case of lysozyme-penicillin-G complex at pH 7.0 which showed a peak of lesser intensity around 290 nm.



Figures 1–4. 1. Binding isotherms for the interaction of lysozyme with penicillin-G at 25°C: *r vs* log [D]. (O), 0.01 M acetate buffer, pH 5.0; (●), 0.01M phosphate buffer, pH 7.0; (Δ), 0.01 M glycine buffer, pH 9.0. **2.** Binding isotherms for the interaction of lysozyme with penicillin-V at 25° C: *r vs.* log [D]. Notations as in figure 1. **3.** Binding isotherms for the interaction of lysozyme with methicillin at 25° C: *r vs.* log [D]. Notations as in figure 1. **4.** Plot of Δ G_r(KJ mol^{-1}) *vs. r* for the interaction of lysozyme with penicillin-G at various pH at 25°C. Notations as in figure 1.

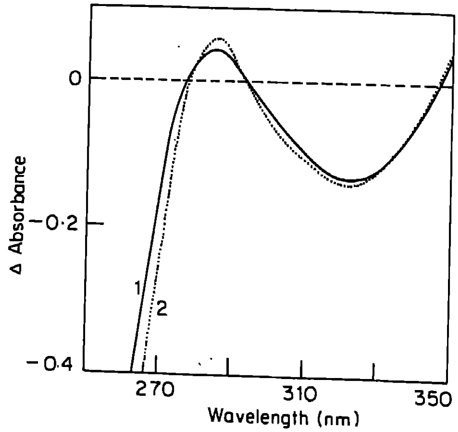


Figure 5. UV difference spectra of 70 μM lysozyme in acetate buffer, pH 5.0 produced by penicillin-G. Concentration of penicillin-G: 1) 9 mM; 2) 11.25 mM.

Since the penicillins differ only in the nature of their side chains, only penicillin-V and methicillin exhibit intramolecular hydrogen bonding between the side chain and the amide group of penicillin nucleus due to stereochemical considerations. This effect of hydrogen bonding would render these compounds more

Table 1. Interaction of lysozyme with penicillins.

Buffer	pH	Penicillin-G			Penicillin-V			Methicillin		
		Number of binding sites ‘n’	$\Delta G_{r,\,\max}$ (KJ mol^{-1})	K	Number of binding sites ‘n’	$\Delta G_{r,\,\max}$ (KJ mol^{-1})	K	Number of binding sites ‘n’	$\Delta G_{r,\,\max}$ (KJ mol^{-1})	K
Acetate buffer (0.01 M)	5.0	4	−14.760	387.3	4	−15.010	428.5	4	−15.070	439.5
Phosphate buffer (0.01 M)	7.0	3	−14.150	303.2	3	−14.500	349.9	3	−14.800	393.6
Glycine buffer (0.01 M)	9.0	2	−13.670	249.5	2	−14.000	285.8	2	−14.040	290.6

hydrophobic (Bird and Marshall, 1967). Therefore, penicillin-V and methicillin exhibit a higher binding strength compared to penicillin-G as the latter exhibits lower hydrophobicity since no intramolecular hydrogen bonding is possible in it. So the binding strength at any given pH is found to be in the order: penicillin-G<penicillin-V<methicillin. This order is in accordance with our ΔG_r values which are in agreement with those obtained in the case of penicillin-G by other workers (Mitsumori *et al.*, 1980).

The appearance of a peak in the aromatic region of the UV difference spectra of all the penicillin complexes at pH 5.0 and only in the case of penicillin-G complex at pH 7.0 indicates the tryptophan residues as the binding sites involving hydrophobic interactions, in agreement with the observations of earlier workers (Mitsumori *et al.*, 1980). In addition, electrostatic interaction of carboxyl group of penicillins will occur with the cationic residues such as lysines or arginines of the enzyme. That these cationic sites for ionic interactions could be only lysines and not arginines has been shown by Philips *et al.* (1970) from their binding studies of penicillins with lysozyme in which the lysines are modified chemically. Since all the penicillins dissociate into carboxylate anions with pKa values around 2.7 (Rapson and Bird, 1963), these ion binding of penicillins with lysozyme would be occurring at all pH used in this investigation. The non-involvement of tryptophans at pH 9.0 with all penicillins and with penicillin-V and methicillin complexes at pH 7.0, indicates that the binding observed here might be largely due to ionic interactions.

The CD spectra of lysozyme-penicillin complexes at pH 5.0 in the near UV region of 320–250 nm show a slight decrease) in their aromatic positive bands from those of native lysozyme with the increase in the concentration of penicillins in the complex (figure 6A)*. A similar trend was observed at pH 7.0 and 9.0 in this region with all the complexes, but the effect was much less pronounced than at pH 5.0.

In the far UV region of 250–200 nm, the magnitude of the molar ellipticity $[\theta]_{222\,\mathrm{nm}}$ for lyozyme-penicillin complexes at pH 5.0 increased with the increase in concentration of penicillins in the complex, indicating an increase in the α-helical content of native lysozyme (figure 6B)*. The effect of binding on the increase in α-helical content was more with penicillin-G than with penicillin-V and methicillin at pH 5.0. This increase in the α- helical content generates a more ordered structure since some of the residues supposed to be involved in the interaction lie in the non-helical region of the tertiary structure. Similar trends were observed at pH 7.0 and 9.0 for the complexes of all the penicillins with lysozyme, but only to a lesser degree.

The CD spectra of lysozyme-penicillin complexes in the near UV and far UV regions show that the penicillin-G at pH 5.0 and 7.0 bind at the tryptophan residues, probably Trp-108 lying at the entrance to the cleft of the hydrophobic box and/or Trp-123 lying on the molecular surface involving hydrophobic interactions, and also at the lysine residues, possibly Lys 96, 97 or 116, the former two

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* In the CD spectra of penicillin complexes with lysozyme, the effect of binding was more at pH 5.0 than at other pH, and that too with penicillin-G complex. Hence, only the CD spectra of penicillin-G complex in the near UV and far UV regions are given in the figure 6A and B respectively.

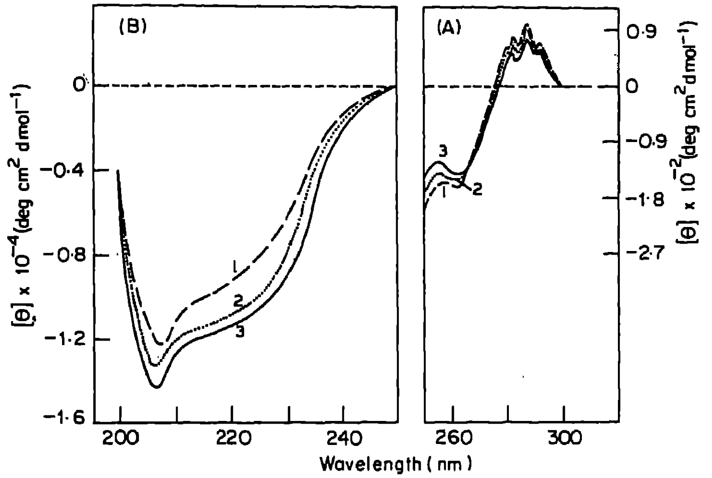

Figure 6. A. Effect of penicillin-G on the CD spectrum of lysozyme in acetate buffer, pH 5.0 (320–250 nm).

Concentration of penicillin-G: 1) 0, M; 2) 0.25 mM; 3) 0.50 mM.

B. Effect of penicillin-G on the CD spectrum of lysozyme in acetate buffer, pH 5.0 (250–200 nm).

Concentration of penicillin-G: 1) 0, M; 2) 0.125 mM; 3) 0.25 mM.

lying at the entrance to the cleft of the hydrophobic box and the latter on the molecular surface involving electrostatic interactions. But all the penicillins at pH 9.0 and only penicillin-V and methicillin at pH 7.0 bind at these lysine residues involving only ionic interactions.

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A new DNA-cloning vector for *Haemophilus influenzae* Rd.

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Abstract. A new, high-efficiency, DNA-cloning vector pJ1-8 was derived in two steps from the chimeric plasmid pD7 consisting of RSF 0885 (*amp*^{r}) and *Haemophilus influenzae* chromosomal DNA. pJ1-8 has only one *Eco*RI site and a molecular weight of only 2.5×10^6. No detectable *amp*^{r} transformation was obtained with pJ1-8 DNA. However, *amp*^{r} transformation increases markedly if *Haemophilus influenzae* chromosomal DNA segments are spliced into it, providing a very facile assay for detecting inserts.

Keywords. Cloning vector; *Eco*RI site; efficient insert detector.

Introduction

A small, multicopy plasmid RSF0885 has been utilized as a vector for cloning DNA in *Haemophilus influenzae* (Setlow *et al.*, 1981; Notani, 1981; Notani *et al.*, 1981). RSF0885 by itself transforms *H. influenzae* with a very low efficiency for the *amp*^{r} marker that it contains. Plasmid RSF0885 has a single *Pvu*II site; cleavage of which with the enzyme generates blunt ends. However, ligation of *Pvu*II-cut *H. influenzae* chromosomal DNA to RSF0885 increases the *amp*^{r} transformation 100 to 1000-fold presumably because RSF0885 does not contain the required 11 base-pair uptake sequence (Sisco and Smith, 1979) which is provided by the spliced chromosomal DNA. A chimeric plasmid pD7 ($M_r \sim 12 \times 10^6$) containing a large chromosomal DNA insert in RSF0885 was isolated and characterized (Notani, 1981). pD7 is cut at 4 sites with *Eco*RI. All the sites are presumably in the chromosomal DNA. A new cloning vector pJ1-8 was derived from pD7 in two steps by reducing the number of *Eco*RI sites to one. This cloning vector was even smaller than RSF0885 and by itself did not transform *H. influenzae*. However, ligation of chromosomal DNA segments increased the *amp*^{r} transformation significantly. Thus, practically each transformant colony contained a chromosome insert. We report the isolation and characterization of a new vector pJ1-8 and other derivatives from pD7.

Materials and methods

Bacterial strains

Haemophilus influenzae Rd (pD7) contains plasmid pD7, which is a spliced chimeric plasmid containing RSF0885 and chromosomal DNAs in the size ratio of approximately 1 : 3, respectively. Other derived strains are described in this paper.

Abbreviations used: M_r, Molecular weight; BHI, brain heart infusion; BSA, bovine serum albumin; DTT, dithiothereitol; DEAE, diethyl aminoethyl.

Media, growth and storage conditions

H. influenzae was grown in 3.7% Difco brain heart infusion (BHI) broth at 37° C with supplements of nicotinamide adenine dinucleotide (NAD) and hemin. Cells were made competent by aerobic-anaerobic-aerobic method (Goodgal and Herriott, 1961). Cultures grown from single colony isolates were stored in 15% glycerol at −73° C.

DNA isolation and purification

Chromosomal DNA was extracted according to the procedure of Marmur (1961). Plasmid DNA was isolated by the method of Hirt (1967) with minor modifications (Notani, 1981). To remove chromosomal DNA, purification was done by ethidium bromide-CsCl equilibrium density-gradient centrifugation (Radloff *et al.*, 1967) or by sedimenting through sucrose gradients (Notani, 1981). For [^{3}H]-labelling of DNA, 8 ml of cells were grown in BHI with 30 μCi [^{3}H]-thymidine (Sp. act. 20 Ci/mM, source Bhabha Atomic Research Centre, Bombay) added per ml.

Restrictions nucleases, DNA cutting and ligation

Restriction nucleases were purchased from Bethesda Research Laboratories, Gaithersburg, Maryland, USA. T4 ligase was a gift from Dr. S. Mitra.

DNA (1–1.5 μg) digestion was done in *Eco*RI reaction mixture (100 μl) containing 50 mM NaCl,100 mM Tris-HCl pH 8.0, 6 mM $MgCl_2$, 50 μg/ml bovine serum albumin (BSA), 1 mM dithiothreitol (DTT) and 2 units of *Eco*RI. Plasmid DNA digestion was carried out at 37° C for 1 to 1.5 h and that of chromosomal DNA at the same temperature for 20 min. Plasmid and chromosomal DNAs were mixed in the ratio of 1:3 respectively. Ligation mixture contained ligase salts (6 mM KCl, 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 1 mM DTT) and ligase additions (1 mM ATP pH 7.0, 10 μg/ml BSA, 10 mM DTT 1 mM spermidine). A maximum of 2 units of T4 DNA ligase was added to the ligase mixture containing 2μg of DNA and kept at 14° C overnight.

Agarose gel electrophoresis

One per cent agarose gels were used in all the experiments. Gel electrophoresis was carried out in Tris acetate buffer pH 7.9 to 8.2 for about 3 h (4 V/Cm). Both the gel and buffer contained ethidium bromide (0.1 μg/ml). Gels were visualized under an ultra-violet (UV) (350 nm) lamp. Photographs were taken with a 35 mm camera using UV filter.

Electro-elution of DNA fragment

Electro-elution of DNA fragment from agarose gel and purification of eluted DNA was carried out as described in Schleif and Wensink, 1981 (pages 124 and 94) except that Tris acetate buffer instead of Tris borate buffer was used for DNA elution from the gel.

Measurement of [^{3}H]-labelled DNA binding to competent cells

Transformation mixture for this purpose consisted of the following: 0.1 ml [^{3}H]-labelled DNA (big or small fragment) + 0.1 ml competent cells + 0.8 ml BHI broth. The mixture was incubated at 37° C for 10 min. 0.5 ml of each mixture was washed 2X in cold and resuspended in 0·1 ml standard saline citrate (SSC). The entire content was spotted on Whatman 3 MM paper discs, treated with trichloroacetic acid, dried and counted in a Beckman scintillation counter.

Estimation of plasmid DNA concentration

This was done as described in Schleif and Wensink (1981) by making a set of standards and visually comparing them with the samples.

Results

*Eco*RI-digested *H. influenzae* chromosomal DNA was ligated with *Eco*RI-cut pD7 DNA. Ligated mixture was used to transform *H. influenzae* ampicillin-sensitive cells. Ampicillin-resistant colonies were picked and grown individually. Lysates were prepared and screened for the presence of altered plasmids. Only one out of ten isolates showed plasmid smaller than pD7. One smaller plasmid, designated pJ1, was characterized further. Figure 1 shows DNA profiles of pJ1 and pD7 sedimented through 5–20% sucrose gradients. pD7 sediments faster than pJ1. pJ1

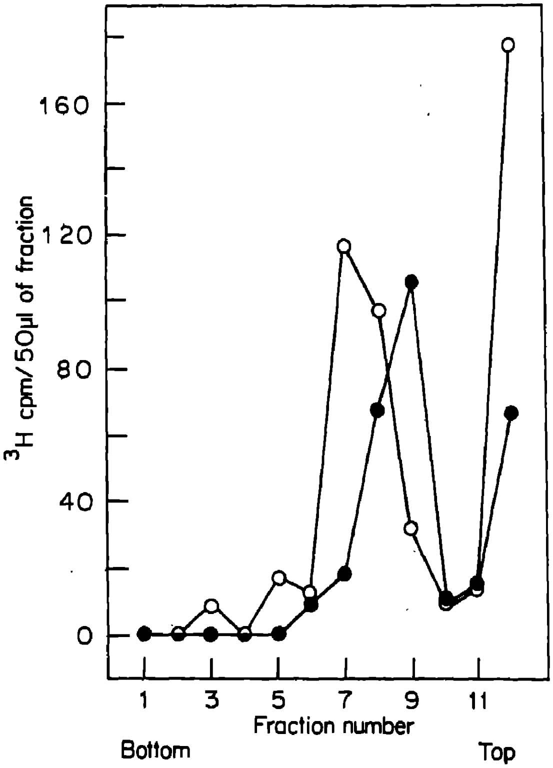

Figure 1. pD7 (○), and pJ1 (●), DNA sedimentation profiles through 5 to 20% sucrose gradients. Centrifugation done at 108500 *g* for 180 min at 20° C.

transforms with a somewhat lower efficiency than pD7 (table 1). When pJ1 DNA was cut with *Eco*RI it gave only two bands indicating that pJ1 has ony two *Eco*RI sites. Molecular weight (M_r) of these two *Eco*RI bands was determined by running *Hpa*-cut T7 DNA standards (McDonell *et al*, 1977) in parallel in the agarose gels (figure 2). The M_r of two fragments was estimated to be 3.9×10^6 and 2.5×10^6 respectively.

Table 1. Transformation (amp^r) activity of pD7 and pJ1 DNAs.

DNA source	DNA (ng/ml)	Amp^r transformants 10^{-4} ml
pD7	100	504
	10	107
	1	8
pJ1	100	153
	10	38
	1	4

pD7 or pJ1 DNA was isolated from 200 ml culture grown under identical conditions. DNA was purified by passing through sepharose 4B column. DNA concentration was estimated by matching intensity of the band with DNA bands of known concentrations (Schleif and Wensink, 1981).

Since *Haemophilus* transformation requires the presence of an 11-bp uptake sequence in the input DNA, it was of interest to determine which of the two fragments contained the required sequence. [^{3}H]-Labelled plasmid DNA in the two bands was separated by gel electrophoresis and electroeluted separately. Both the bands were then purified by passing through diethylaminoethyl (DEAE)-sephacyl ion exchange column. The two fragments were separately mixed with competent cells. After washing, binding of acid-insoluble [^{3}H]-label to the cells was determined (table 2). There was little binding of smaller fragment whereas the bigger fragment was bound substantially.

Construction of EcoRI-chromosomal library

Chromosomal DNA from wild-type *H. influenzae* cell culture was digested with *Eco*RI and ligated to *Eco*RI-cut pJ1 DNA. Competent cells were exposed to (i) uncut pJ1 DNA, or (ii) *Eco*RI-cut pJ1 or (iii) *Eco*-RI cut but self-ligated pJ1 or (iv) *Eco*RI cut pJ1 and *Eco*RI cut chromosomal DNA ligated mixture. Transformation mixture was plated and amp^r colonies were scored. Uncut pJ1 gave the highest transformation but *Eco*RI-cut plasmid yielded no transformants. Self-ligated pJ1 DNA restored the transformation to about 7% of the uncut plasmid and pJ1 ligated to chromosomal DNA to less than 2%. pJ1 DNA concentration was the same (250 mg) in all the cases.

Ampicillin-resistant colonies obtained from the transformation with ligated chromosomal DNA were picked and clear lysates were made to determine the size of the plasmid. Altogether 28 clones were analysed by agarose gel electrophoresis. Out of 28 clones, 19 were found to carry plasmids either smaller or bigger than pJ1. Thus we conclude that at least about 70% of the plasmids are other than pJ1

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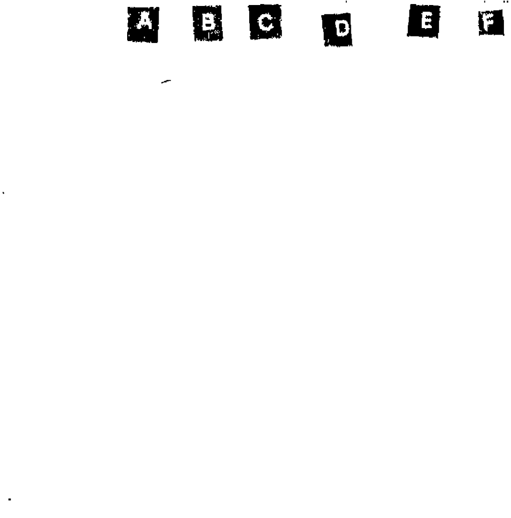

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Figures 2 and 3. 2. Electrophoresis patterns of pJ1 DNA, lane A — two prominent bands show CCC form of plasmid DNA (the fastest moving) and OC form (the slowest moving). *Eco*RI-digested pJ1 lane B — two bands which correspond to the 1st and the 4th slowest bands of *Hpa*-I digested T7 DNA standards in lane C (McDonnel *et al*, 1977) with molecular weights of 3.9×10^6 and 2.5×10^6 respectively. **3.** Electrophoresis patterns of *Eco*RI-cut pJ1-8, pJ1-26 and pJ1 DNA (lanes A, C, E respectively) and uncut pJ1-8, pJ1-26 and pJ1 (lanes B, D, F respectively).

Table 2. Binding of *Eco*RI-cut pJ1 DNA label by competent *H. influenzae* Rd cells.

Fragments	cpm/0.1 ml (acid insol.)		Transformants
	Input	Uptake	(*amp*^{r})
Big fragment			
Experiment I	300	239	0
Experiment II	436	116	0
Small fragment			
Experiment I	120	14	0
Experiment II	211	10	0

whereas all of the 10 *amp*ʳ clones from self-ligated plasmids DNA showed the presence of a plasmid of the same size as the original pJ1.

Three of the 19 recombinant plasmids were studied further. These three plasmids were designated as pJ1-8, pJ1-18 and pJ1-26. pJ1-26 is the largest of the three (figure 3 lane D) and transforms most efficiently (table 3). pJ1-8 gives little or no [^{3}H]-DNA binding to the competent cells as well as no transformation (table 3) and is the smallest in size (figure 3 lane B). Upon splicing with *H. influenzae* chromosomal DNA *amp*ʳ transformants could be scored by plating even at 10^{-4} dilution of the transformation mixture. pJ1-18 and pJ1-26 are both bigger than pJ1 and pJ1-8 is smaller than pJ1. Molecular weight of pJ1-8 was estimated to be about 2.5×10^6. Figure 3 shows *Eco*RI-cut pJ1, pJ1-8 and pJ1-26 electrophoresed through agarose gels. pJ1 and pJ1-26 each yield 2 bands but pJ1-8 gave only a single band. Both pJ1 and pJ1-26 have one band corresponding in size to pJ1-8.

Table 3. Efficiency of newly-isolated, chimeric plasmid DNA's to transform *H. influenzae* cells.

DNA source	Uptake of DNA cpm/0.1 ml	*amp*ʳ transformants/ 10^{-4} ml
pJ1	1116	18
pJ1-18	1902	58
pJ1-26	1184	84
pJ1-8	10	0 (10^{-2})

Discussion

Data in tables 1 and 3 indicate that there is variability in transformation efficiency amongst different plasmids. This could be due to the size of the chromosomal insert, since *rec* 1 and *rec* 2 gene expression is required for transformation with chimeric plasmids (Setlow *et al*, 1981). pJ1-8 gives little or no transformation but upon splicing with *H. influenzae* chromosomal DNA the increase is substantial. Thus it fulfils the main criterion of efficient and facile screening for plasmid carrying inserts as it was in the case of RSF0885 cut with *pvu*II (Setlow *et al*, 1981). Moreover, it is cut only once with *Eco*RI which produces 'sticky' ends unlike *Pvu*II which cuts once into RSF0885 but produces blunt ends. It may be anticipated that ligation would be more efficient with sticky ends.

Molecular weight of RSF0885 is 3.7×10^6 whereas that of pJ1-8 is only2.5×10^6. It is apparent that RSF0885 has suffered a deletion in the non-essential region. *Eco*RI site, not present in the original plasmid RSF0885 may have been generated with the deletion or more likely a bit of chromosomal DNA with *Eco*RI site may still be present. From low transformation (nearly zero) it may be deduced that pJ1-8 does not have an uptake site. At any rate, pJ1-8 works as a very efficient vector for screening and cloning *H. influenzae* chromosomal DNA inserts.

Acknowledgements

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RNA polymerase activity in isolated nuclei of *Nicotiana sanderae* callus: Characteristics and modulation during differentiation

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Abstract. Isolated nuclei from differentiating cultures of *Nicotiana sanderae* showed increased levels of RNA polymerase activity as compared to the nuclei from callus cultures. The RNA synthetic activity was dependent on nucleotide triphosphates and Mg^{2+} and was destroyed by RNase. Maximum acitivity was obtained in the presence of 50 mM $(NH_4)_2SO_4$ and α-amanitin inhibited 40% and 55% of the activity in the nuclei from callus and differentiating tissue respectively. The nuclei from differentiating tissue elicited a 3-fold increase in RNA polymerase I and a 4-fold augmentation in RNA polymerase II activities.

Keywords. *Nicotiana sanderae*; RNA polymerase; phytohormones; differentiation.

Introduction

Phytohormones play an important role in root and shoot formation from callus cultures and during seed germination. Although the biochemical events preceding differentiation are not clearly understood, during the development of embryo into root and shoot, enhanced syntheses of RNA and proteins occur (Davies and Larkins, 1973; Chen *et al*, 1975). In a variety of developing systems, regulation of gene expression has been studied mainly at the transcriptional level (Key 1969; Duda, 1976; Becker, 1979). Nuclear RNA polymerase activity levels undergo dramatic changes during developmental stages such as seed germination (Guilfoyle and Key, 1977) and regeneration of organs (Yu, 1975). However, there are no reports on the modulation of nuclear RNA polymerase activities in a differentiating system. Therefore, attempts have been made to study the regulation of gene expression, specifically at the transcriptional level, in the differentiating callus of *Nicotiana sanderae*. In this communication, we report the characteristics of RNA polymerase activities in the isolated nuclei and the changes in their levels during differentiation.

Materials and methods

Chemicals

2,4-Dichlorophenoxy acetic acid (2,4-D), naphthalineacetic acid (NAA), benzyl

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Abbreviations used: 2,4-D, 2,4-Dichlorophenoxy acetic acid; NAA, naphthalene acetic acid; BAP, benzyl aminopurine; MS medium, Murashige and Skoog's medium; MES, 2-(N-morpholinoethanesulphonic acid); GM, grinding medium, 5 mM MES-NaOH, pH 6.1, 4 mM magnesium acetate, 5 mM, 2 mercaptoethanol, 0.25 M sucrose, 4% gum arabic and 1% Triton X-100.

aminopurine (BAP), Trizma base, 2-(N-morpholinoethane sulphonic acid) (MES), Triton X-100 and nucleotide triphosphates were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. [^{3}H]-UTP (54 Ci/mmol) was from Radiochemical Centre, Amersham, England, the nylon sieves were obtained as a gift from Dr. Gigot, CNRS, Strasbourg, France, and α-amanitin was obtained as a gift from Dr. J. R. Tata, National Institute for Medical Research, Mill Hill, London. All other chemicals were of analytical grade.

Explants

The callus cultures initiated from shoot explants were grown in Murashige-Skoog's (MS) medium (Murashige and Skoog, 1962) supplemented with 1 mg/L 2,4-D and shoot differentiation was obtained in MS medium containing 1 mg/L NAA and 2 mg/L BAP. The cultures were grown at 25–27° C, under 16/8 h light-dark cycles for about 3 weeks.

Isolation and purification of nuclei

Best nuclear preparations, both in terms of yield and transcriptional activity were obtained using the procedure suggested by Dr. Gigot, Institute de Biologie Moleculaire et Cellulaire, CNRS, Strasbourg, France (Personal communication). The tissue was homogenized in a Waring blendor at full speed for 1 min with 3 g/10 ml grinding medium (GM)-5 mM MES-NaOH, pH 6.1, 4 mM magnesium acetate, 5 mM 2-mercapto-ethanol, 0.25 M sucrose, 4% gum arabic and 1% Triton X-100. The homogenate was filtered through 4 layers of cheese cloth, 1 layer of lint cloth and passed through a 100 μ nylon screen. The filtrate was centrifuged at 450 *g* for 15 min and the pellet was suspended in GM without gum arabic and was agitated during 20 min and then filtered through a 25 μ nylon screen and subsequently through a 10 μ nylon screen. The filtrate was centrifuged through a 1.2 M sucrose cushion in GM (without gum arabic) at 450 *g* for 15 min. The pellet was suspended in GM (without gum arabic), and was washed three times with the same buffer and the nuclear pellet was suspended in buffer containing Tris-HCl, pH 8.0, 0.25 M sucrose; 4 mM $MgCl_2$ and 5 mM 2-mercaptoethanol. The nuclei thus prepared was free of carbohydrate contaminations, unbroken cells, fragmented particles etc. As observed under phase contrast microscope, the nuclear-preparations were essentially free of membranous material.

When the nuclei were isolated in the presence of the hormones, all the buffers contained the respective hormones used for the growth (1 mg/L 2,4-D for callus and 1 mg/L NAA as well as 2 mg/L BAP for differentiating tissue) of the cultures.

RNA polymerase assay

The assay was a slight modification of the procedure used by Mennes *et al.* (1977) for tobacco nuclei. Reaction was carried out at 26° C for 20 min in a final volume of 200 μl and the reaction mixture contained the following: Tris-HCl, pH 8.0, 40 mM; $MgCl_2$, 5 mM; EDTA, 0.1 mM; 2-mercaptoethanol, 1 mM; KCl 150 mM; bovine serum albumin, 125 μg; ATP, GTP and CTP, 0.3 mM each, unlabelled UTP, 0.02 mM; [^{3}H]-UTP, 10 μCi and nucelar suspension containing 10^5–10^7 nuclei.

At the end of the incubation period, unlabelled UPT was added to a final concentration of 10 mM to dilute out the unincorporated radioactivity (which was earlier found to stick to the RNA unspecifically) and the RNA was precipitated by the addition of 3 ml of ice-cold 5% trichloroacetic acid containing 1% sodium pyrophosphate. The suspension was allowed to stand for 30 min to 1 h and the precipitate was washed (by centrifugation) four times with cold 5% trichloroacetic acid containing pyrophosphate, and once with enthanol. The final pellet was taken in 100 µl of 0.3 N NaOH and incubated at 37° C for 14–16 h, and was subsequently neutralized with perchloric acid. The suspension was counted in 10 ml of aqueous scintillation mixture (250 ml Triton X-100, 500 ml toluene and 3 g 2-diphenyloxazole).

DNA measurements

The nuclei were processed according to Mennes *et al.* (1977) and DNA was estimated by the diphenylamine method as suggested by Giles and Myers (1965).

Results

The kinetics of total nuclear RNA polymerase activity were measured in the nuclei from undifferentiated callus and callus induced to undergo differentiation for 24 h. The rate as well as the extent of RNA synthesis in the nuclei from the differentiating tissue was higher than that from the control callus (figure 1). This could result from more number of RNA polymerase molecules initiating transcription *in vivo*, either due to an initiating-specificity alteration or to an increased number of enzyme molecules.

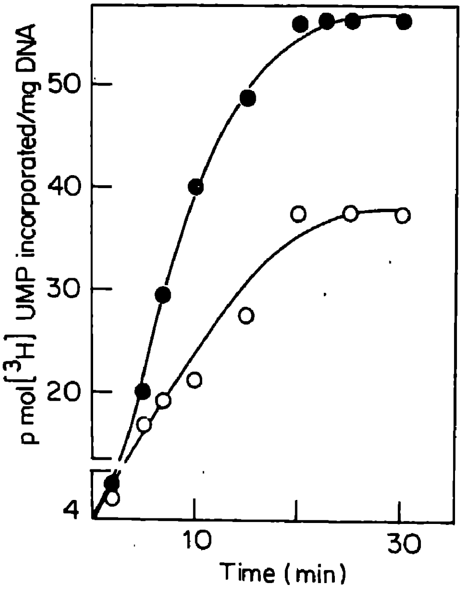

Figure 1. Kinetics of nuclear RNA transportation. (○), Callus; (●), diferentiating tissue.

In callus nuclei, 38 µmol of [^{3}H]-UMP residues were incorporated per mg nuclear DNA, which increased to 56 µmol per mg DNA in the nuclei of differentiating tissue. However, various factors such as the source of plant material, growth conditions, composition of the isolation media, method of homogenization etc, contribute to the RNA synthetic capacity of the nuclei (Mennes *et al.*, 1977).

The divalent cation (Mg^{2+}) concentration for total nuclear RNA polymerase activity was optimal at 5 mM, after which there was a decline in the activity (figure 2). It was observed that maximum activity was obtained at a concentration of about 5×10^6 nuclei in a reaction volume of 200 µl.

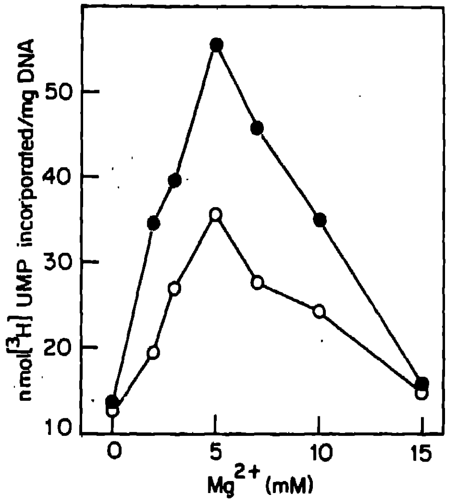

Figure 2. Effect of Mg^{2+} concentration on total nuclear RNA polymerase activity. (○), Callus; (●), differentiating tissue.

The characteristics of the nuclear transcription are given in table 1. In the absence of any one of the nucleotide triphosphates, the activity was approximately 30% and marginal in the absence of Mg^{2+}. The differences in the residual activity of nuclear polymerase(s) from callus and differentiating tissues observed in the absence of one of the unlabelled nucleotide triphosphates could be due to the differences in the levels of endogenous nucleotide pool. In the presence of RNase, more than 90% of the labelled product was destroyed.

Table 1. Characteristics of RNA polymerase activity.

	Total RNA polymerase activity*	
System	Callus tissue	Differentiating tissue
Complete	94.5	409.2
−ATP	34.0	114.0
−GTP	31.0	114.0
−CTP	31.0	113.0
+RNase	4.7	31.0
$-Mg^{2+}$	10.9	12.0

**p* mol [^{3}H]-UMP incorporated/mg DNA.

The callus (24 h) nuclei were isolated in the presence of 1 mg/L 2,4-D and those from differentiating tissue (24 h) in the presence of 1 mg/L NAA and 2 mg/L BAP.

The effect of ionic strength on nuclear RNA polymerase activity was tested (figure 3). The activity was maximum at 50 mM $(NH_4)_2SO_4$ and this activity is assumed to be largely contributed by RNA polymerase I and to a very less extent by the other polymerases (Duda, 1976). At higher concentrations of $(NH_4)_2SO_4$ (150–200 mM), RNA polymerase II is maximally active, whereas RNA polymerase III and I could contribute only to a very low percentage of the activity observed (Duda, 1976).

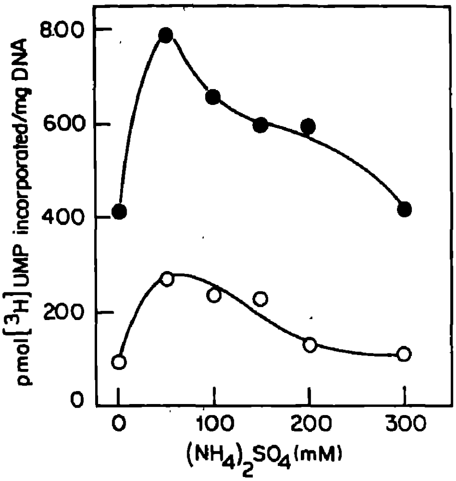

Figure 3. Effect of ionic strength on nuclear RNA polymerase activity. The nuclei were isolated in the presence of the hormones. (○), Callus; (●), differentiating tissue.

Table 2. Effect of α-amanitin on nuclear RNA polymerase activity.

Tissue	Concentration of α-amanitin	RNA polymerase activity[a]
Callus	0	225.0
	0.1	176.0
	1.0	168.0
	2.0	156.0
	5.0	140.0
	10.0	135.0
Differentiating	0	590.7
	0.1	579.0
	1.0	360.0
	2.0	343.8
	5.0	315.3
	10.0	265.0

[a] *p* mol of [^{3}H]-UMP incorporated/mg DNA.

The inhibition by α-amanitin at various concentrations was tested. The nuclei were isolated in the presence of the hormones. Assays were done in the presence of 150 mM ammonium sulphate, at various concentrations of α-amanitin. The assay mixture with ammonium sulphate and α-amanitin was preincubated for 10 min after which [^{3}H]-UTP was added. The per cent inhibition at the highest concentration of α-amanitin (10 mM) was 40% and 55% in callus and differentiating tissue respectively..

The effect of α-amanitin, a potent inhibitor of eukaryotic transcription, on total nuclear RNA polymerase was studied. Because α-amanitin inhibits RNA polymerase II activity, its effect was tested in the presence of 150 mM $(NH_4)_2SO_4$. The results showed that 55% of the total activity was inhibited by 10 μg/ml α-amanitin in the case of nuclei from differentiating tissue, as against 40% inhibition of nuclei from callus tissue (table 2).

RNA polymerases I and II in the nuclei were quantitated by means of their differential sensitivity to α-amanitin, as well as their altered ionic strength requirements (Widnell and Tata, 1966; Hentschel and Tata, 1977). RNA polymerase II is measured by subtracting the residual activity in the presence of α-amanitin at 150 mM $(NH_4)_2SO_4$ from total activity obtained at 150 mM $(NH_4)_2SO_4$ (without α-amanitin). The total activity in the presence of 50 mM $(NH_4)_2SO_4$ (without α-amanitin) represents RNA polymerase I activity. It was observed that the differentiating tissue contained elevated levels of both the polymerases and that the enhancement in RNA polymerase II was higher (4 times) than that of RNA polymerase I (3 times) activity (table 3).

Table 3. RNA polymerases I and II in the nuclei of callus and differentiating cultures.

Tissue	RNA polymerase I	RNA polymerase II
	p-mol [^{3}H]-UMP incorporated/mg DNA	
Callus	267.9	84
Differentiating	793.8	322

The amounts of RNA polymerases I and II present in the nuclei were quantitated by virtue of their differential sensitivity to α-amanitin (as given in the text) and by the effect of $(NH_4)_2SO_4$ requirements. The nuclei were isolated in the presence of the hormones.

Discussion

The present studies on RNA synthesis in *N. sanderae* cultures show that differentiation of callus into shoot is preceded by an increased RNA polymerase activity and this enhancement is reflected in the activities of RNA polymerase I as well as II. It has long been known that analogous systems such as hormone-treated seedlings, and plant organs elicit increased RNA content and *in vitro* RNA polymerase activity as compared to their control counterparts (Key, 1969; Chen *et al.*, 1975). Studies using carrot suspension cultures (Sengupta and Raghavan, 1980; Fujimura *et al.*, 1980) showed that the content as well as the rate of synthesis of RNA increases during somatic embryogeny. Similarly, *N. sanderae* cultures show raised levels of RNA synthesis at very early stages of differentiation (24h). This could be due to the increased levels of nuclear RNA polymerases observed at 24 h after induction of differentiation.

Sekiya and Yamada (1978) observed that when tobacco cells cultured *in vitro*, were induced to differentiate into shoots, there was an initial decrease followed by an enhancement in the soluble RNA polymerase activity. However, a 3-4 fold enhancement in the activities of nuclear RNA polymerases I and II was elicited by the differentiating callus of *N. sanderae* during early stages. Although evidence indicates that there is an overall increase in the synthesis of all species of RNA as a response to hormone-induced developmental changes, reports from several laboratories indicate that RNA polymerase I is mainly involved (Guilfoyle and Hanson, 1973; Chen *et al*, 1975; Rizzo *et al*, 1977). However Guilfoyle *et al* (1980) have recently observed a 10-fold and a 6-fold augmentation in RNA polymerases. I and II respectively as a consequence of treatment with 2,4-D on soybean tissue. Similarly, Burkhanova *et al* (1980) have also reported that BAP could bring about an increase in RNA polymerases I and II activities in the nuclei from barley protoplasts by a factor of 2.6 to 2.8 and 2.2 to 2.5 respectively. The present study shows that the nuclei from tissues that were induced to differentiate by treatment with NAA and BAP, contain 3-fold and 4-fold high levels of RNA polymerases I and II respectively. This could be due to increased enzyme synthesis, activation of preexisting RNA polymerases, changes in the compartmentation of free and chromatin-bound enzyme, increased template availability, acting either alone or in combination. In an attempt to distinguish between these possibilities, we have demonstrated that there is a shift in the ratio of chromatin bound/free enzyme molecules occurring during differentiation (to be published elsewhere). However, to understand the modulation of the RNA polymerases, their fractionation and subsequent analysis of the transcription products are required.

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On the presence of sulphate in pituitary lutropin

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Abstract. The presence of sulphate in the carbohydrate of pituitary lutropin from different species has been investigated using a biosynthetic approach. Pituitaries from rats, rabbits, goats, and buffaloes were incubated in the presence of $^{35}SO_4^=$ and the $^{35}SO_4^=$-labelled proteins in the tissue immunoprecipitated with a well characterized anti-sheep lutropin serum. The incorporation into immunoreactive lutropin was low in the case of rat, rabbit and goat pituitaries while, it was considerable in the case of buffalo pituitaries. Hence further characterization studies were carried out on $^{35}SO_4^=$-labelled proteins of buffaloes. The physico-chemical, immunological and biological properties of radio-labelled buffalo pituitary material were shown to be similar to those of standard lutropin. In *in vitro* conditions of incubations, most of the incorporation of $^{35}SO_4^=$ was observed into tissue lutropin while under similar conditions of incubation, [^{14}C]-amino acids were found to get incorporated mostly into medium lutropin. The physiologically specific releasing hormone, lutropin-releasing hormone was found to stimulate the release of $^{35}SO_4^=$-labelled lutropin from the rabbit pituitaries into the medium. These results give indirect evidence that sulphate could be present in pituitary lutropin.

Keywords. Sulphate incorporation; pituitary hormone; lutropin; gonadotropin; immunoprecipitation.

Introduction

Structural work on the carbohydrate portion of bovine pituitary lutropin (LH) has revealed the presence of sulphate on the terminal hexosamine residues (Parsons and Pierce, 1980). This was based on direct spectrophotometric estimation of sulphate released from the hormone by acid treatment. Supporting evidence for the presence of sulphate moiety in the oligosaccharide chains of LH has come from metabolic studies in which radioactive $^{35}SO_4^=$ has been shown to be incorporated into pituitary LH (Hoshina and Boime, 1982; Kalyan Rao and Bahl, 1983; Rajyalakshmi *et al.*, 1983). The significance of this sulphate moiety is not known. The placental glycoprotein hormone human chorionic gonadotropin (hCG) on the other hand does not have any acid labile sulphate (Parsons and Pierce, 1980).

We are interested in the study of regulation of secretion of LH (Muralidhar *et al.*, 1977). We have characterised the biosynthetic precursor of bovine pituitary α-subunit (Muralidhar and Bahl, 1981) with an idea of locating regulatory sites in the

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Abbreviations used: LH, Luteinizing hormone or lutropin; LH-RH, lutropin-releasing hormone; hCG, human chorionic gonadotropin; ECF, ethyl chloroformate; a/s, antiserum; NRS, normal rabbit serum; Da, Dalton (molecular weight); KRBG, Krebs-Ringer Bicarbonate Buffer with 0.2% glucose pH 7.4.

enzymatic pathway leading to the secretion of intact LH. In an extension of this study we recently observed that radioactive $^{35}SO_4^=$ could get incorporated into immunoreactive LH from rat and buffalo (bovine) pituitaries (Rajyalakshmi *et al.*, 1983). We undertook the studies reported here in order to obtain more convincing evidence for this and to determine whether sulphate is present in LH of other species as well as to probe whether sulphation/desulphation have any role to play as storage/secretion signals.

Materials and methods

Hormones, chemicals, animals and antiserum

Lutropin (NIH-LH-S18) and LH-RH were gifts from the National Institute of Arthritis, Metabolic and Digestive Diseases (NIAMDD), NIH, Bethesda, USA. [^{14}C]-Algal protein hydrolysate (Sp. activity 20–30 mCi/m.atom) and $^{35}SO_4^=$ (Carrier free) were purchased from Bhabha Atomic Research Centre, Trombay, Bombay, India. Bovine serum albumin (BSA), Aprotinin and Thimerosal (Grade II) were obtained from Sigma Chemical Company, St. Louis, Missouri, USA. Dulbecco's modified Eagle's medium (DMEM) was bought from GIBCO laboratories, Grand Island, USA. Freund's complete adjuvant was purchased from DIFCO laboratories, Detroit, USA. All other chemicals were of AnalaR grade.

Antiserum to NIH-LH-S18 was obtained by immunizing healthy male adult rabbits against LH by the procedure of Vaitukaitis *et al.* (1971). Blood was collected from an ear vein, serum separated, decomplemented and stored frozen with 0.01% azide as a preservative. The rabbit was periodically boosted with a saline solution of LH. The antiserum was purified by absorption with 1 : 10 diluted normal sheep serum (NSS) to remove nonspecific antibodies against tissue and serum antigens. The cross-reaction of this antiserum with pituitary LH of different species was checked by Ouchterlony double diffusion test (Moudgal and Li, 1961). An immunoadsorbent was prepared from this antiserum by cross linking the antiserum proteins by the use of the bifunctional reagent, ethyl chloroformate (ECF). The procedure was essentially same as that of Avrameas and Ternynck (1967).

Pituitary incubations

Pituitaries were quickly removed from rats (castrated a week earlier), rabbits, goats, or buffaloes at the time of slaughter, cleansed and were cut into quarters or sliced. Pituitaries in all cases were ready for incubation within 1–2 h after killing of animals. These were pooled and randomly distributed into incubation flasks. Incubations were conducted for 2–3 h in a metabolic shaker in either DMEM medium or a modified Krebs-Ringer Bicarbonate buffer (KRB) containing $MgCl_2$ instead of $MgSO_4$, 0.2% glucose and 0.2% BSA, where necessary neutralized [^{14}C]-algal protein hydrolysate (5 μCi/ml) or $^{35}SO_4^=$ (25–100 μCi/gland) were included.

Processing of tissue and medium

The tissue, at the end of the incubation was rinsed three times with the KRBG buffer to get free of incubation medium and then homogenised in KRBG buffer. The total radioactive proteins were precipitated with 10% trichloroacetic acid. The precipitate was washed 3 times with cold trichloroacetic acid and dissolved in 0.05 N NaOH and the aliquots counted for radioactivity.

For immunoprecipitation, two procedures were followed. In one, aliquots of homogenate after centrifugation (9000 *g*/15 min/4° C) were incubated with antiserum (a/s) or normal rabbit serum (NRS) for 1 h at 37° C followed by three days and nights at 4° C. Protease inhibitor (Approtinin) was included during immunoprecipitation. The immunoprecipitate was washed with chilled saline, dissolved in 0.05 N NaOH and aliquots counted for radioactivity. The media were similarly processed except that they were dialysed extensively and lyophilised before processing for trichloroacetic acid precipitation or immunoprecipitation. In the second procedure a water insoluble polymer of a/s or NRS proteins was used instead of antiserum or normal rabbit serum (Avrameas and Ternynck, 1967).

Chromatography and electrophoresis

The chromatography of $^{35}SO_4^-$-labelled proteins on SP-Sephadex was done essentially according to the procedure of Papkoff *et al.* (1965). Electrophoresis was performed in 10% gels essentially according to the procedure of Reisfeld *et al.* (1962). At the end of the run, gels were removed, proteins fixed and cut into 2 mm slices. The slices were dissolved in H_2O_2 and counted for radioactivity using 10 ml of Bray's solution (Bray, 1960). Standard sheep LH was also electrophoresed and stained with Coomassie blue.

Bioassay and immunoassay

The biological activity of standard lutropin and the [^{35}S]-labelled material from bovine pituitaries was tested by the Parlow's ovarian ascorbic acid depletion assay (Parlow, 1961). The ascorbic acid content of the ovaries was estimated according to the procedure given in Oser (1976). The immunological identity of LH and [^{35}S]-labelled material was tested by Ouchterlony double diffusion test (Kabat and Mayer, 1972).

Results and discussion

Sulphated proteins in pituitary glands of different species

The specific activity of the sulphated pituitary proteins in different species is given under table 1. Thus the rat pituitary proteins were labelled to the highest specific activity among the species investigated in this study while the buffalo pituitary proteins had the lowest specific activity (table 1). This could be due to different number of sulphated glycoproteins in different animals or due to existence of variation in the degree of sulphation within a limited number of sulphated glycoproteins. From results published by Rosa and Zanini (1981) and Kalyan Rao and Bahl (1983) it could be seen that at least 6–8 sulphated proteins are made by rat and sheep pituitaries respectively (counted from their figures).

We had earlier shown that increasing the concentration of $^{35}SO_4^-$ in the medium had no effect on the specific activity of the tissue protein (Rajyalakshmi *et al.*, 1983). Supporting evidence for the organic nature of the bound sulphate was the observation that when the sulphated material is incubated in hot acid (4 M HCl

Table 1. Pituitary halves or quarters or slices were incubated with $^{35}SO_4^-$ in modified KRBG buffer for 3 h. The tissues were processed for total TCA precipitable radioactivity. See Methods section for details. Results are average of two experiments only.

Species	Pituitaries per flask	Approximate weight of the pituitaries (mg)	$^{35}SO_4^-$ in the medium (μCi/ flask)	Radioactivity TCA (cpm/mg protein)	Precipitated Antiserum* (cpm/flask)
Rat	5	1	25	32,000	71
Goat	2	150	200	24,000	143
Rabbit	2	3	200	15,390	184
Buffalo	2	1000	200	3,020	4,040

*Radioactivity precipitated with LH a/s minus the radioactivity precipitated with normal rabbit serum.

in boiling water bath), no radioactivity was retained in the material (data not shown). Others have shown that exhaustive pronase digestion of the $^{35}SO_4^-$-labelled material results in quantitative recovery of label in the oligosaccharide chains (Kalyan Rao and Bahl, 1983).

Immunoprecipitation of $^{35}SO_4^-$-labelled proteins of rat, rabbit, goat, and buffalo pituitaries

When the $^{35}SO_4^-$-labelled proteins, synthesized under *in vitro* incubations of pituitaries with $^{35}SO_4^-$ were subjected to immunoprecipitation with the well characterised anti-sheep lutropin serum (Rajyalakshmi *et al.*, 1983), all the species studied gave positive results of incorporation of $^{35}SO_4^-$ into immunoreactive LH (table 1). In the case of goat pituitaries, an immunoadsorbent was used instead of a/s. The degree of incorporation was however different in these species. In the case of the rat, rabbit and goat, the incorporation was marginal or low, while significant amounts of radioactivity was incorporated into buffalo pituitary LH (table 1). When computed as per cent of total trichloroacetic acid precipitable radioactivity the value ranged from 0.1% in the case of goat to 3% in the case of rabbit calculated on a mg protein basis. The specificity of the immunoprecipitation was established as described earlier (Rajyalakshmi *et al.*, 1983). The studies of Rosa and Zanini (1981) and Kalyan Rao and Bahl (1983) have reported failure to detect incorporation of $^{35}SO_4^-$ into rat pituitary LH using autoradiographic techniques. Our results, where immunoprecipitate was directly taken for radioactive counting show marginal incorporation. Hence it might be too low for autoradiographic detection. However, Hoshina and Boime (1982) have been successful in demonstrating $^{35}SO_4^-$ incorporation into rat pituitary LH. The discrepancy between the results of Hoshina and Boime (1982), Rosa and Zanini (1981) and Kalyan Rao and Bahl (1983) is difficult to explain.

Similarities between [^{35}S]-labelled buffalo pituitary protein and LH

In order to obtain more convincing data on the presence of sulphate in LH, buffalo pituitary glands were incubated with $^{35}SO_4^-$, and [^{35}S]-labelled proteins were subjected to certain purification procedures. We have earlier established that these steps of purification which are essentially those of Papkoff *et al.* (1965) yield a homogeneous preparation of buffalo LH (T. Rajendrakumar, A. S. Arunasmithasri and K. Muralidhar — unpublished work). Thus, the [^{35}S]-labelled buffalo pituitary proteins were extracted with 0.15 M $(NH_4)_2SO_4$ solution, nonspecific proteins precipitated with metaphosphoric acid and the supernatant made 50% saturated with respect to $(NH_4)_2SO_4$. The 50% $(NH_4)_2SO_4$ precipitate was dialysed and subjected to immunoprecipitation. About 10% of this radioactivity could be precipitated as [^{35}S]-labelled immunoreactive LH. When the same 50% $(NH_4)_2SO_4$ precipitate after dialysis, was chromatographed on a SP-Sephadex column in 0.01 M Na_2-HPO_4, 20% of the [^{35}S]-labelled proteins was held up on the column and could be eluted with 0.05 M Na_2-HPO_4. These are exactly similar to the conditions under which LH is purified reinforcing the idea that pituitary LH possibly has sulphate moiety.

The [^{35}S]-labelled material from the 50% $(NH_4)_2SO_4$ precipitate was checked for immunological and biological activity of LH. As shown in table 2, the material was immunologically active against anti-sheep LH serum and biologically active in ovarian ascorbic acid depletion assay, specific for LH (Parlow, 1961). When subjected to electrophoresis in 10% acrylamide gels, the major band of radioactivity from among the $^{35}SO_4^=$-labelled proteins of the 50% $(NH_4)_2SO_4$ precipitate was found to comigrate with the Coomassie blue stainable LH band (figure 1). Hence these results indicate that the $^{35}SO_4^=$-labelled buffalo pituitary protein(s) has physico-chemical, immunochemical and biological properties similar to those of standard sheep LH. These results do also indicate that there are other sulphated proteins in the pituitary which behave like LH during purification at least upto the SP-Sephadex chromatography step. Similar results are obtained in the case of sheep pituitary glands incubated with $^{35}SO_4^=$ (T. Rajendrakumar, A. S. Arunasmithasri and K. Muralidhar — unpublished work).

Table 2. Immunological and biologica properties of $^{35}SO_4^=$-labelled buffalo pituitary protein fraction.

	Ouchterlony test*	Bioassay† (ascorbate/100 mg ovary)
Saline	–	93 ± 25
NIH-LH-S20	+	52 ± 5
$^{35}SO_4^=$ material	+	34 ± 9.9

* Against rabbit anti-sheep lutropin.

† Ovarian ascorbic acid depletion assay.

Differential labelling of tissue and medium LH

In our studies using [^{14}C]-amino acids as precursors for the synthesis of immunoreactive rat LH, we found that most of the incorporated radioactivity was found in the medium with negligible amounts in the tissue (data not shown). This observation that newly synthesized hormone was secreted preferentially agrees with the

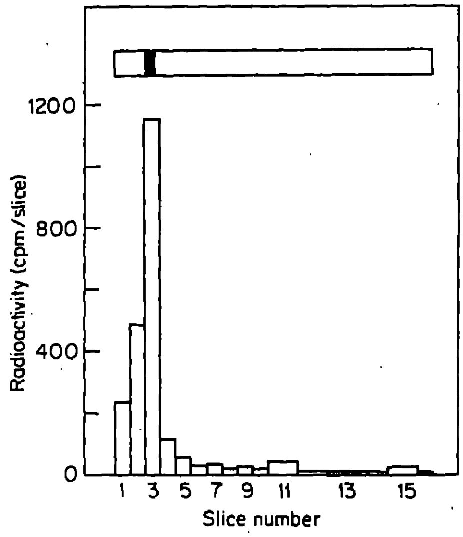

Figure 1. Buffalo pituitary slices were incubated with $^{35}SO_4^{=}$. The slices were then homogenised and the homogenate subjected to purification steps as described by Papkoff *et al.* (1965). The 50% $(NH_4)_2SO_4$ precipitate was subjected to electrophoresis in a 10% polyacrylamide gel according to Reisfeld *et al.* (1962). Standard LH (100 µg) was run in another gel and stained with Coomassie Blue. The radioactive gel was sliced and the slices counted for radioactivity.

observations of other workers (Labrie *et al.*, 1971). Under similar conditions of incubation with $^{35}SO_4^{=}$ as precursor, we found most of the incorporation into tissue LH with negligible amounts into medium LH (table 3). This is in contrast to what we observed in the case of [^{14}C]-amino acids. Hoshina and Boime (1982) reported recently that they could measure $^{35}SO_4^{=}$-labelled free α-subunit in the medium of bovine pituitary incubations while detection of $^{35}SO_4^{=}$-labelled β-subunit or whole LH was reported to be difficult. In the studies of Kalyan Rao and Bahl (1983), the immunoreactive LH and $^{35}SO_4^{=}$-labelled protein from the medium did not co-chromatograph on Sephadex G-100, while from the tissue both behaved identically on Sephadex G-100. Most of the medium radioactivity was eluting at a posi-

Table 3. Pituitary quarters were incubated with $^{35}SO_4^{=}$ modified KRBG buffer for 2 h. The tissue and media were separately processed for immunoreactive LH. See Methods for details. Results are average of two experiments only.

Species	Pituitaries per experiment	$^{35}SO_4^{=}$ in the medium (µCi/flask)	Radioactivity precipitated* (cpm)	
			Tissue	Medium
Rat	25	800	1950	Nil.
Rabbit	2	200	400	85

*Radioactivity precipitated with LH a/s minus radio activity precipitated with NRS.

tion characteristic of α-subunit of LH. Our antiserum against whole LH has no significant cross reaction with α-subunit (data not shown) and hence we cannot measure this constituent specifically in our experiments. While in our studies we measured radioactivity directly in the immunoprecipitates, Hoshina and Boime (1982) tried to detect $^{35}SO_4^=$-labelled LH, α-subunit or β-subunit by electrophoresis and autoradiography subsequent to immunoprecipitation. In view of the low degree of incorporation into medium constituents, it is not surprising that they could not measure with ease [^{35}S]-labelled medium LH or β-subunit.

Effects of LH-RH

We have consistently observed that the incorporation of $^{35}SO_4^=$ into medium LH was negligible. That the specific activity of medium LH is much lower than tissue LH has been observed by others also (Kalyan Rao and Bahl, 1983). We thought it would be interesting to enhance the release of the hormone by LH-RH. In these experiments, rabbit pituitary quarters were first incubated with $^{35}SO_4^=$ for 2 h, rinsed free of initial medium and incubated in a fresh medium free of radioactivity in the absence or presence of fixed dose of LH-RH for an additional 30 min. The content of $^{35}SO_4^=$-LH in the tissue and media was measured. The content of free sulphate in tissue and media was also measured. We found that while there was no significant increase in $^{35}SO_4^=$ incorporation into tissue LH, LH-RH caused enhanced release of $^{35}SO_4^=$-LH into the medium (figure 2). When free $^{35}SO_4^=$ was measured in tissue and media and the results expressed as ratio of medium radioactivity over tissue radioactivity, LH-RH was found to cause a consistent drop in the ratio at all the doses tested (table 4). These results would indicate that LH-RH could have enhanced the rate of utilization of $^{35}SO_4^=$ for sulphation. However, this has to be confirmed by actual assay of the enzymes involved in the activation and incorporation of sulphate into oligosaccharides of LH.

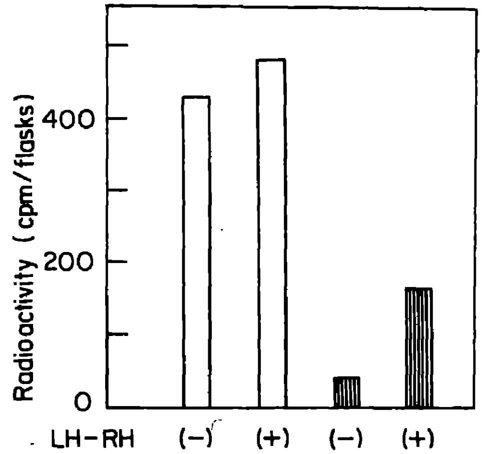

Figure 2. Rabbit pituitary quarters were incubated first in the presence of $^{35}SO_4^=$. At the end of 2 h, they were rinsed free of radioactivity and incubated for an additional 30 min in radioactivity free KRBG buffer pH 7.4. At the end of the incubation, $^{35}SO_4^=$-labelled LH was measured in tissue and medium. Results of a representative experiment is given, (□), $^{35}SO_4^=$-labelled LH in the tissue; (▥), $^{35}SO_4^=$-labelled LH in the medium.

Table 4. Rabbit pituitary quarters were incubated with $^{35}SO_4^-$. They were then incubated in a fresh radioactivity free medium in the presence of LH-RH. Total radioactivity was measured in tissue and media.

LH-RH (ng/ml)	Radioactivity (cpm)		M/T
	Tissue (T)	Medium (M)	
0	99660	215090	2.1
100	92100	99000	1.07
400	125480	152370	1.2
1600	64680	105160	1.6
3200	76280	111200	1.4

Evidence for the presence of sulphate in pituitary LH has come from two approaches. In one, direct estimation of acid released sulphate from the hormone was undertaken (Parsons and Pierce, 1980). In the second approach metabolic studies indicated that $^{35}SO_4^-$ could get incorporated into pituitary immunoreactive LH (Hoshina and Boime, 1982; Kalyan Rao and Bahl, 1983; Rajyalakshmi *et al.*, 1983). The latter evidence would still be circumstantial. Recently, Kalyan Rao and Bahl (1983) reported the purification of $^{35}SO_4^-$-labelled protein from sheep pituitaries incubated with $^{35}SO_4^-$ to apparent homogeneity and the establishment of the exact location of bound sulphate. In both our present report and the studies of Kalyan Rao and Bahl (1983), the absolute biological identity (100% recovery) of $^{35}SO_4^-$-labelled pituitary protein with LH has not been proved while physicochemical and immuno-chemical similarities between the two have been demonstrated. One way to do this would be to prove that $^{35}SO_4^-$-labelled pituitary protein purified to homogeneity is active fully in receptor binding assays. Our present results, showing that an agent like LH-RH which is known to enhance the release of immunoreactive LH from pituitaries could, also cause enhanced release of $^{35}SO_4^-$-labelled material (which was precipitated by the same antiserum), is a physiological proof that pituitary LH could have sulphate.

The role of this sulphate moiety is not known at the present. It is possible that it might serve as a signal for storage of the hormone. Thus there might be two pools of LH, one for immediate secretion, and another for storage and secretion in response to acute demand. The latter pool may represent the sulphated portion. We do not as yet know of any role for sulphate either in contributing to plasma half life or in hormone action. It could also contribute to the structural heterogeneity of LH like sialic acid does in the case of hCG.

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Transcription in ultra violet-irradiated plasmodia of *Physarum polycephalum*

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Abstract. In *Physarum polycephalum*, transcription is significantly inhibited for the whole of the ultra violet-induced, extended G2 phase. Although, there is some increase in the rate of transcription closer to the first post-irradiation mitosis, thereafter, ovrall synthesis continues to be low. Also, the plasmodia developed significant resistance to actinomycin D during this period. The resistance is both in terms of mitosis and RNA synthesis. The overall depression in transcription and the drug resistance are apparently related phenomena.

Keywords. Ultra violet-irradiation; transcription; actinomycin-resistance.

Introduction

Nuclear divisions in the multinucleate, syncytial plasmodia of *Physarum polycephalum*, a myxomycete, are synchronous and rhythmic in nature. Fusion of several microplasmodia grown in agitated cultures would yield large disc-shaped surface plasmodium (macroplasmodium) of several centimetres in diameter, containing millions of nuclei in a single mass of cytoplasm. A huge surface plasmodium, for all practical purposes, is a single (multinucleate) giant cell and because of the large size is ideal for biochemical analysis. Moreover, synchrony of mitosis is also exhibited between sister macroplasmodia made from pooled microplasmodial cultures and this is yet another advantage offered by this organism. The plasmodium is extraordinarily resistant to radiations because of the multinucleated state. This fact and the synchronous and rhythmic mitotic cycles make the organism ideal for studying cycle-dependent radiation effects other than mere cell killing and also in providing information on mitotic triggering mechanisms. Several investigations have already been carried out to understand the role of macromolecular synthesis in the control of mitotic cycles in *Physarum*, employing ultra violet light as a perturbing agent (Sachsenmaier, 1966; Devi *et al*, 1968a; Sachsenmaier *et al*, 1970; Devi and Guttes, 1972; Dworzak and Sachsenmaier, 1973; Sachsenmaier and Dworzak, 1976; Tyson *et al*, 1979; Valsala Kumari and Nair, 1981). Some of the above studies as well as those by Sudbery and Grant (1975, 1976) have helped in the formation of various models on the rhythmicity of the mitotic cycles in this organism (Reviewed by Winfree, 1980).

Abbreviations used: UV, Ultra violet; PFM, post-fusion mitosis; PIM, post-irradiation mitosis; SDM, semi-defined medium; SDS, sodium dodecyl sulphate; CR, control ribosome; CN, control nuclei; IR, irradiated ribosome, IN, irradiated nuclei.

Ultra violet (UV)-irradiation of the plasmodia causes the degeneration of large number of nuclei and the nucleo-cytoplasmic ratio is thus altered. Because of this, the delayed first post-irradiation mitosis (PIM) is followed by several shorter-than-normal mitotic cycles (Devi *et al*, 1968a; Sachsenmaier *et al*, 1970; Devi and Guttes, 1972). It is also known that the irradiated plasmodia become largely resistant to actinomycin in terms of mitosis (Devi and Guttes, 1972) and RNA synthesis (Dworzak and Sachsenmaier, 1973; Sachsenmaier and Dworzak, 1976). With these findings in view, we have now made further studies on transcription in the irradiated plasmodia during the extended G2 phase prior to the first post-irradiation mitosis and also during the following short mitotic cycle.

Materials and methods

Culturing the organism

The Wisconsin strain (M_3 C) of *Physarum polycephalum* was grown as microplasmodia in suspension cultures on a semi-defined medium (SDM) (Daniel and Baldwin, 1964), at a temperature of 24° C. Mitotically synchronous surface plasmodia (macroplasmodia) were made by fusion of a large number of microplasmodia according to the method of Guttes and Guttes (1964). Sectors from the same plasmodium or sister plasmodia made from pooled microplasmodial suspension, and hence showing synchronous mitosis among themselves, are used in the different experiments. Surface plasmodia in G2 prior to the second post-diffusion mitosis (PFM) or in G2 prior to the third PFM are used, especially in regard to DNA replication synchrony is best after the first PFM.

Determination of mitotic stages

The mitotic stages were determined by observation of ethanol fixed smear preparations made from explants of plasmodia removed at different times in the mitotic cycle, under the oil immersion lens of a phase microscope (Guttes *et al*, 1961).

Irradiation, actinomycin treatment and labelling

For irradiation, a Philips 15-watt germicidal lamp was employed which emits approximately 90% of its UV energy at 2537 Å at a dose rate of $9.37 \cdot Jm^{-2}$ Sec^{-1}. Total dose of irradiation was 1400 Jm^{-2} except where otherwise mentioned.

Actinomycin D (Sigma Chemical Co., St Louis, Missouri, USA) was used at a concentration of 200 μg/ml of SDM. It is known from earlier studies that, in *Physarum*, actinomycin D is required to be used at these rather high concentrations for any significant effect in, *in vivo* experiments (Mittermayer *et al*, 1965), although much lower concentrations are sufficient *in vitro*, while working with isolated plasmodial nuclei (Mittermayer *et al*, 1966b; Grant, 1972). [^{3}H]-Uridine (BARC, India; Specific activity, 20.5 Ci/mmol) was used for *in vivo* labelling of RNA and details are provided as legends to the respective figures and tables.

Autoradiography

Small explants from labelled plasmodia were fixed in acetic acid : alcohol (1 : 3) or Champy's fixative and autoradiography was carried out on 3 μ sections of the material. Acid soluble components were removed by washing the slides with 5%

cold trichloroacetic acid for 15 min. Kodak AR-10 stripping film was used in the preparation of autoradiographs. Exposure was for 12 days at 4° C and development was for 12 min in Kodak D-19 Developer at 20° C. Visual grain counting under the oil immersion lens of a phase contrast microscope was carried out for obtaining quantitative data. In determining the number of grains over the nucleus, all grains over the nucleus and those within a distance of approximately 1 μ from the nuclear periphery, were counted as nuclear label. Slides used for grain counting had very low background. Nevertheless, background corrections have been made to obtain the values given in the different figures.

Preparation of nuclei and ribosomes

Nuclei and ribosomes were prepared by adopting the techniques used by Mittermayer *et al.* (1966a) as well as some aspects of the methods by Zellweger and Braun (1971). The plasmodia, after labelling with [^{3}H]-uridine at desired times during the mitotic cycle, were given a wash in SDM and were homogenised in an electrically operated teflon-pestled-glass homogeniser. The homogenisation medium used was the following: 0.2 M sucrose, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 M Tris-HCl pH 7.2. The homogenate was centrifuged at 50 *g* in a SS 34 rotor of a Sorvall RC2-B Centrifuge for 10 min. The nuclear pellet obtained after centrifugation of the supernatant at 2800 *g* was washed by spinning through 1 M sucrose in 0.01 M $MgCl_2$, 0.01 M Tris-HCl pH 7.2 and later kept frozen. The supernatant got after removal of the nuclei was again centrifuged at 5000 *g* for 15 min in order to remove mitochrondria, membrane fragments etc. To the supernatant obtained after this centrifugation, sodium dodecyl sulphate (SDS) was added to give a final concentration of 0.1% and mixed thoroughly. This was then centrifuged through a discontinuous gradient of a 20% sucrose solution (10 ml) layered over 10 ml of a 40% sucrose solution at 100,000 *g* for 2 h in an SW 27 rotor of a Beckman L5-50 ultracentrifuge. The sucrose solutions were prepared in the following buffer: 0.8 M KCl, 10 mM $MgCl_2$, 10 mM Tris-HCl pH 7.2. The ribosomal pellets thus obtained were then stored frozen.

Before extracting RNA from the nuclear and ribosomal fractions, each was treated with 0.25 M cold (4° C) perchloric acid for 15 min. Following this, the suspension containing nuclei were centrifuged at 2800 *g* in a SS 34 rotor of a Sorwall RC 2-B centrifuge for 15 min. The suspension containing ribosomes were centrifuged at 100,000 *g* for 1 h in an SW 27 rotor of a Beckman L5-50 model ultracentrifuge. Washing with 0.25 M perchloric acid in the cold was repeated once more. The supernatant from the second wash was used for obtaining background counts.

Extraction and estimation of RNA

For the removal of acid soluble components and extraction of acid-insoluble RNA from the plasmodia, the procedure of Mittermayer *et al.* (1964) was followed. Plasmodial sectors were shaken in cold (4° C) 4% trichloroacetic acid in acetone-water (1 : 1) mixture for about 10 min on a vortex. The suspension was then left in the cold at 4° C for 12 h. After this the plasmodial pieces were resuspended by shaking again on a vortex and centrifuged at 200 *g* in an IEC PR-6 centrifuge. The residue obtained was treated once more with the above trichloroacetic acid : acetone mixture, followed by 3 washes in 0.25 M perchloric acid at 4° C. The supernatant from the last wash was used for obtaining background counts.

RNA was extracted from whole plasmodial residue obtained after the above acid washes or from the nuclear and ribosomal fractions respectively, by using bovine pancreatic ribonuclease. For this extraction, for every 1.9 ml of 0.06 M $KHCO_3$, 0.1 ml of a 1 mg/ml solution of RNase (RNase used here was a product of Boehringer, W. Germany and the stock solution, prepared in distilled water, was kept in boiling water bath for 10 min to inactivate contaminating DNase) was added to the respective smaples. Incubation was carried out at 30° C for 1.5 h. The reaction was stopped by adding 0.5 ml of a solution of 0.5 M trichloroacetic acid for every 2 ml of the extract. From each sample, aliquots were taken for radioactivity assay and for RNA estimation. For radioactivity assay, aliquots were spotted on millipore filters and counted in a Beckman LS 100 C Liquid Scintillation Counter, using a toluene, PPO, POPOP mixture. Quantitative estimations on RNA were made by employing the orcinol test (Ceriotti, 1958).

Results

Both autoradiographic (figure 1) and biochemical (table 1) studies show that nuclei of irradiated plasmodia incorporate much less [^{3}H]-uridine, following a pulse-labelling schedule, when compared to control. For example, autoradiographs show that in the irradiated plasmodia, there is a shift in the labelling pattern from the higher grain classes of nuclei to the lower and there is considerable increase in the percentage of nuclei with no grains as well (figure 1). While this is the situation for most of the extended G2 phase, nuclear labelling increases considerably closer to the first PIM and thereafter (figure 2B and table 1). Ribosomal labelling also increases following the first PIM (table 1). However, the ratio of the specific activity of RNA from ribosomes to that of nuclear RNA in the irradiated plasmodia (IR/IN), taken during this period, indicates that the increase in ribosomal labelling is not proportional to the increase in nuclear label. This ratio was even lower before the first PIM (table 1).

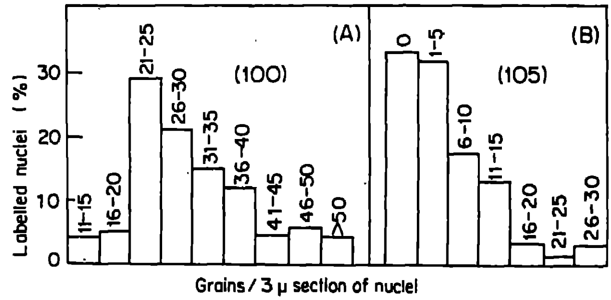

Figure 1. RNA synthesis in irradiated and control plasmodia as measured by autoradiography (dose of irradiation: 1267 Jm^{-2}). Abscissa: grains/3 μ section of nuclei: ordinate: % of labelled nuclei. Pulse labelling for 10 min with [^{3}H]-uridine, 30 μCi/ml of salts medium (Daniel and Baldwin, 1964). A and B are sectors of the same macroplasmodium.

A—Control, labelled 49 min prior to the second PFM.

B—Irradiated 49 min prior to the second PFM and labelled immediately after that. Value given in parenthesis is the number of nuclei counted.

Table 1. Specific activity of RNA in nuclear and ribosomal fractions from UV-irradiated plasmodia of *Physarum polycephalum*.

Time of labelling (column 1)	cpm/μg RNA after pulse and chase: Nuclei, Control (column 2)	Nuclei, Irradiated (column 3)	Ribosomes, Control (column 4)	Ribosomes, Irradiated (column 5)	CR/CN (column 6)	IR/IN (column 7)
1 h 45 min before third PFM (15 min after irradiation)	45786	15526 (66.09)	14886	4658 (68.71)	0.33	0.30
45 min before third PFM (1 h 15 min after irradiation)	55966	13605 (75.69)	16335	5330 (67.37)	0.29	0.39
3 h. before first PIM (6 h after irradiation)		38319 28011		6331 3930		0.17 0.14
4 h 45 min after first PIM or 3 h 30 min before second PIM (13 h 45 min after irradiation)		36719 56246		9492 14774		0.26 0.26

CR, Control ribosome; CN, control nuclei; IR, irradiated ribosome; IN, irradiated nuclei.

Controls are given for the first two experimental material. No strictly comparable controls are possible for others. Values given in parenthesis denote % inhibition in relation to respective controls. Pulse labelling with 100 μCi/ml of [^{3}H]-uridine for 30 min followed by 1 h chase in SDM.

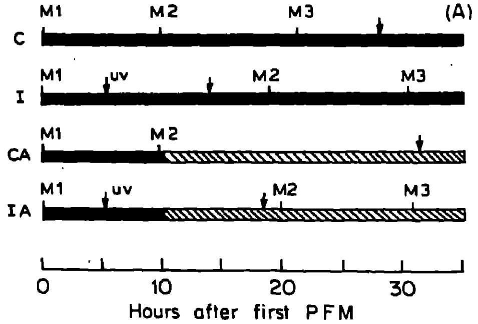

Figure 2. A. The schedule of irradiation, actinomycin treatment and [^{3}H]-uridine labelling, in relation to mitotic cycle, in the plasmodium, the labelling data of which are given in figure 2B.

C, Unirradiated sector on SDM; I—irradiated sector on SDM; CA—unirradiated sector on actinomycin; IA—irradiated sector on actinomycin; M1—first PFM, M2—second PFM (first PIM in the case of irradiated sector) M3, third PFM (second PIM in the case of irradiated sector) ↓ UV—marks the time of irradiation. ■—marks the duration of actinomycin treatment. ↓ Marks the beginning of labelling with [^{3}H]-uridine.

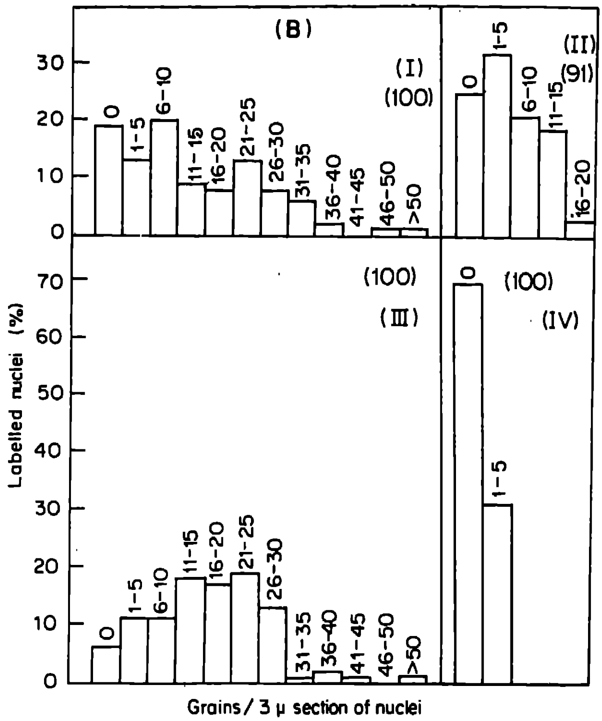

Figure 2B. Effect of actinomycin D on RNA synthesis in irradiated plasmodia as measured by autoradiography (dose of irradiation: 1267 Jm^{-2}). Abscissa: grains/3 µ section of nuclei: Ordinate: % of labelled nuclei. Concentration of actinomycin – 200 µg/ml of SDM. Pulse labelling – 10 min; Concentration of [^{3}H]-uridine – 30 µCi/ml in salts medium. I, II, III and IV are sectors of the same plasmodium.

I, Irradiated on SDM; II, irradiated on actinomycin; III, control on SDM; IV, control on actinomycin.

Value given in parenthesis is the number of nuclei counted.

In support of our earlier studies (Devi and Guttes, 1972) and that of Sachsenmaier (Dworzak and Sachsenmaier, 1973; Sachsenmaier and Dworzak, 1976), we have observed that the process of mitosis and the duration of the mitotic cycle in the irradiated plasmodia is not susceptible to rather high doses of actinomycin D (200 µg/ml), to which control plasmodia are highly susceptible. For example, it is seen that additional mitotic delay due to the effect of this drug is much less when plasmodia irradiated during early G2 are placed on the drug a few hours after irradiation (figures 2A and 3A). This cannot be due to the lack of time for the action of the drug, as in these experiments, the plasmodia are continuously exposed to the drug for the whole of the UV induced, extended G2 phase of over 8 h. In unirradiated control plasmodia, similar treatment leads to several hours of delay towards mitosis (figures 2A and 3A and Mittermayer *et al*, 1965) followed by abnormal mitosis (a kind of abortive mitosis) in majority of the nuclei (Devi *et al*, 1968b; Guttes *et al*, 1969). Furthermore, in the irradiated plasmodia, the mitotic cycle following the first PIM is fully resistant to 200 µg/ml of this antibiotic (figures 2A and 3A). This effect is highly significant, as in the unirradiated plasmodia, once a

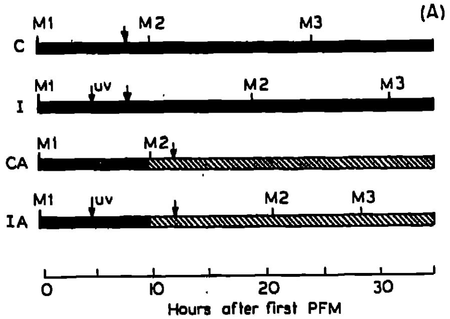

Figure 3A. The schedule of irradiation, actinomycin treatment and [^{3}H]-uridine labelling, in relation to the mitotic cycle, in the plasmodium, the data of which are given in figure 3B. The explanation for the symbols are as same as for figure 2A.

mitosis occurred in the presence of actinomycin D, another mitosis did not occur in the continued presence of the drug (figure 3A). Yet another observation made is that, in cases where the UV induced, extended G2 phase is further increased by actinomycin D, the duration of the following short mitotic cycle is decreased correspondingly, such that the overall period remains about the same, but subject to a certain minimum for the short mitotic cycle (figure 3A). Also, the large number of abortive mitotic figures induced by this antibiotic in control plasmodia (Devi *et al*, 1968b; Guttes *et al*, 1969), are largely absent in similarly treated irradiated plasmodia.

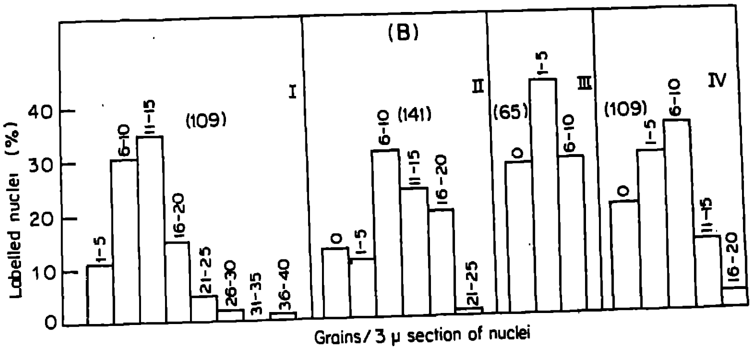

Figure 3B. Effect of actinomycin D on RNA synthesis in irradiated plasmodia as measured by autoradiography (dose of irradiation: 1267 Jm^{-2}). Abscissa: grains/3 μ section of nuclei; Ordinate: % of labelled nuclei. Concentration of actinomycin—200 μg/ml of SDM: Pulse labelling—10 min; concentration of [^{3}H]-uridine—30 μCi/ml in salts medium. I, II, III and IV are sectors of the same plasmodium.

I, Control on SDM; II, irradiated on SDM; III, control on actinomycin; IV, irradiated on actinomycin.

Value given in parenthesis is the number of nuclei counted.

The significantly decreased sensitivity of mitosis to actinomycin D in the irradiated system is accompanied by a corresponding decrease in the action of this drug on the incorporation of [^{3}H]-uridine into RNA. For example, when unirradiated plasmodia in G2 show from 60% to 70% inhibition of [^{3}H]-uridine incorporation after about 2 h of drug treatment, similarly treated irradiated plasmodia show only 26% to 44% inhibition, when compared to untreated irradiated plasmodia (table 2). However, as we can see, irradiation itself causes varying degress of inhibition of transcription in these plasmodia (tables 1 and 2).

Table 2. Effect of actinomycin D on RNA synthesis in irradiated plasmodia of *Physarum polycephalum*.

Time of collection of samples	cpm/µg RNA			
	SDM		Actinomycin D	
	Control	Irradiated	Control	Irradiated
1 h 30 min before third PFM	AC_1-1188	AI_1-939 (21)	AC_2-470 (60)	AI_2-692 (26)
55 min before third PFM	BC_1-1022	BI_2-816 (20)	BC_2-268 (74)	BI_2-455 (44)
9 h after irradiation, during the extended G2 phase prior to first PFM	–	AI_3-389	–	AI_4-585 (no inhibition)
	–	BI_3-704	–	BI_4-849 (no inhibition)
5 h 50 min after first PIM	–	AI_5-390	–	AI_6-737 (no inhibition)
	–	BI_5-323	–	BI_6-351 (no inhibition)

Plasmodia A and B were irradiated 4 h before third PFM. AC_1 and AC_2, control sectors from plasmodium A; AI_1 to AI_6, irradiated sectors from plasmodium A; BC_1 and BC_2, control sectors from plasmodium B. BI_1 to BI_6, irradiated sectors from plasmodium B. Dose of actinomycin D-200 µg/ml; labelling for 30 min with 50 µCi/ml of [^{3}H]-uridine. Values given in parenthesis denote percentage inhibition either due to irradiation or due to actinomycin. For computing percentage inhibitions in irradiated plasmodia treated with actinomycin, counts obtained from irradiated are taken as 100%. For computing percentage inhibitions due to irradiation alone, counts obtained from control are taken as 100%.

Another observation which we have made is that, although, biochemical analysis made on total cellular RNA show that the UV-irradiated system is completely resistant to actinomycin D (200 µg/ml), from the later part of the extended G2 phase onwards (table 2), autoradiographs show that, nuclear labelling shows some susceptibility to the drug even during this period. Some of the higher grain classes of nuclei are absent in the autoradiographs prepared from the drug-treated, irradiated plasmodia, with a corresponding increase in the lower grain classes (figures 2B and 3B). In the biochemical analysis, where specific radioactivity of total cellular RNA is assayed, these smaller level effects, observable in a cytological study of individual nuclei are apparently not detected.

Discussion

In the present studies, we have taken specific activity of RNA following pulse labelling schedule with [^{3}H]-uridine, as an indicator of transcription, for the following reasons. (i) The rate of [^{3}H]-uridine incorporation into RNA is likely to represent true differences in the rate of RNA synthesis in this organism. Using such a method of assay, it has been possible to show the biphasic pattern of overall RNA synthesis during the mitotic cycle of *Physarum* in an *in vivo* system (Mittermayer *et al*., 1964; Braun *et al*., 1968; Sauer *et al*., 1969) which has later been confirmed in *in vitro* studies (Mittermayer *et al*., 1966b; Grant, 1972). (ii) RNA precursor pools do not markedly alter during the cell cycle, with the possible exception of a short period round about mitosis (Grant, 1973). (iii) Following UV-irradiation, there is a significant decrease in total nuclear DNA, as a result of degeneration of many nuclei in this syncytial system (Devi *et al*., 1968a; Devi and Guttes, 1972). Since the number of degenerating nuclei varies in the different plasmodia, evaluating transcription in terms of nuclear DNA would not be as meaningful as it should be. (iv) Our autoradiographic studies, where assay is in terms of incorporation of [^{3}H]-uridine into invidiual nuclei, corroborate much of what is found biochemically.

The resistance to actinomycin D (C$_1$) (200 μg/ml) shown here and elsewhere (Devi and Guttes, 1972; Dworzak and Sachsenmaier, 1973; Sachsenmaier and Dworzak, 1976) could only be partial, as it was observed by Sachsenmaier and Dworzak (1976) that nuclei in the irradiated plasmodia get arrested in metaphase at 500 μg/ml of actinomycin (they had used actinomycin C), an effect seen in control plasmodia at 100 μg/ml to 250 μg/ml of the drug. In control plasmodia, 500 μg/ml of the drug prevents the nuclei even from entering mitosis. In this context, it may be noted that the concentration of actinomycin D required for inhibition of RNA synthesis and mitosis is extraordinarily high even in control plasmodia (Mittermayer *et al*., 1965) and may very well be, in part at least, due to permeability barriers, as RNA synthesis by isolated nuclei in *Physarum* is blocked by actinomycin D at a concentration as low as 0.1 μg/ml (Mittermayer *et al*., 1966b). If this is so, it is possible that irradiation might have increased these barriers and is responsible for the above observed results. We had checked this point in an earlier study using [^{3}H]-actinomycin D incorporation (12.5 μCi/ml; specific activity — 2.9 Ci/mmol; incubation *in vivo* for 1 h), followed by quantitative autoradiography and found no evidence to support this. Also, some of our preliminary *in vitro* RNA synthesis studies employing isolated nuclei from irradiated plasmodia show that these nuclei are less susceptible to actinomycin D inhibitions, when compared with control nuclei (P. A. Valsala Kumari and Vimala R. Nair, unpublished). However, it may still be worthwhile to repeat the *in vivo* incorporation experiments (Devi and Guttes, 1972) for longer incubation periods with higher concentrations of [^{3}H]-actinomycin D.

It is known that actinomycin D reacts preferentially with derepressed regions of DNA (Geuskens, 1977). Any change in the conformation of the DNA molecule should change the degree of affinity of the drug to it, as for maximum binding, there should be perfect matching of the symmetries of either of these molecules (Stryer, 1975). Some conformational changes are to be expected in the DNA of the irradiated system because of the presence of photoproducts such as thymidine dimers formed as a result of UV action (Smith and Hanawalt, 1969). In view of the above, a certain amount of decrease in the effect of actinomycin D on the irradiated plasmodia is expected, as we have shown that UV has considerably inhibited transcription in this system (figure 1 and tables 1 and 2). In fact, it is seen that, if the percentage inhibitions due to UV alone are added up to that obtained by actinomycin in the irradiated system, the combined value is about the same as that obtained by actinomycin alone in the control plasmodia (table 2). It is perhaps relevant here to note that, not only UV, but ionizing radiation also decreases the sensitivity of RNA synthesis and nuclear division to actinomycin C in *Physarum* (Sachsenmaier and Dworzak, 1976).

In a sucrose density gradient analysis of [^{3}H]-uridine pulse-labelled RNA from *Physarum*, we had shown that, there is a decrease in the production of 26 S nd 19 *S* rRNA types in the irradiated plasmodia with a corresponding increase in the synthesis of low molecular weight RNAs including 4 *S* RNA (Valsala Kumari and Vimala Nair, 1981). The lower (in comparison to control in G2) specific activity of total cellular RNA (the bulk of which would be accounted for by rRNA) in the irradiated plasmodia during the extended G2 phase (table 2) even at a time when nuclear labelling has increased (table 1 column 3) and the decreased ribosomes/nuclei label seen during this period (table 1 column 7) are meaningful when viewed in this context.

It is known that, just as in the case of other eukaryotes (Penman *et al*, 1968), rRNA synthesis in *Physarum* is preferentially susceptible to the action of actinomycin D (Mittermayer *et al*, 1964; Grant, 1972). The rDNA in this organism is nearly exclusively contained (except for 5 S genes) in the nucleolus as free extrachromosomal DNA. It has a density of 1.712 ± 0.002 g/ml corresponding to 54% guanine plus cytosine (Seebeck and Braun, 1980). In view of this, the preferential action of actinomycin D on rDNA transcription in *Physarum* is expected as actinomycin is known to interact with the guanine residues of DNA, each of the cyclic peptides of the drug forming hydrogen bonds with the 2-amino group of guanine in the DNA (Stryer, 1975). A consequent decrease in the action of this antibiotic on the irradiated plasmodia is also expected because of the apparent inhibition of rRNA production in this system.

It would appear that the decrease in overall growth (Devi and Guttes, 1972), the decrease in the production of 26 *S* and 19 *S* rRNA types (Valsala Kumari and Vimala Nair, 1981) and the reduced susceptibility to actinomycin D seen in the irradiated plasmodia are all related phenomena.

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Distribution and properties of a 'lectin-like' glycoprotein from leaves and stems of *Dolichos biflorus*: Implications on the role of lectins in plants

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Abstract. Studies on the distribution of the *Dolichos biflorus* lectin during the life cycle of the plant have shown that the seed lectin appears at about 27 days after flowering and is localized in the cotyledons. Although this lectin disappears during the absorption of the cotyledons, a related molecule that cross reacts with antibodies to the seed lectin, appears in the stems and leaves of the plant. Structural studies on this cross reactive material show that it appears to have one subunit in common with the active subunit of the seed lectin and another subunit that has a higher molecular weight than the seed lectin subunits. The subunits of the seed lectin and cross reactive material may all represent different degrees of completion or modification of a common gene product.

The cross reactive material has been found to have carbohydrate binding activity at low ionic strength and a significant amount of this "lectin-like" glycoprotein appears to be associated with the cell wall. Further studies on the distribution and properties of lectins during the life-cycle of the plant may be valuable in obtaining a better insight into the physiological role(s) of these molecules in the plants.

Keywords. Lectin; distribution; structure; role.

Introduction

The abundance of plant lectins and their abilities to specifically recognize particular carbohydrate residues have made them valuable tools for the characterization of complex carbohydrates and cell surfaces. However, despite the vast literature on these molecules and their variety of applications, little is known about their physiological function(s) in the plant.

A variety of roles have been proposed for plant lectins, ranging from their involvement in plant growth by serving as storage or transport proteins (Boyd, 1963), mitogenic stimulators of plant embryonic cells (Howard *et al*, 1972) or involvement in cell wall extension (Kauss and Glaser, 1974) to their use as protective agents (Boyd, 1963) or as attractants for specific strains of *Rhizobia* needed for nitrogen fixation in leguminous plants (Hamblin and Kent, 1973). Although some of these proposed roles have attracted considerable attention in recent years, there is little evidence to confirm any of these roles as a physiological function of lectins. Before such a role can be established it is essential to have more information on the distribution and properties of lectins during the life cycle of the plant. The present paper reviews the studies we have conducted on the distribution and properties of the *Dolichos biflorus* lectins and discusses the possible implications of these studies on the role(s) of lectins in plants.

Properties of *Dolichos biflorus* seed lectin

The seeds of the *Dolichos biflorus* plant contain a lectin that selectively agglutinates blood group A substance (Boyd and Shapleigh, 1954) due to its specificity for terminal nonreducing α-N-acetyl-D-galactosamine residues (Etzler and Kabat, 1970; Hammarström *et al*, 1977). This lectin is a tetrameric glycoprotein with a molecular weight of approximately 110,000 (Carter and Etzler, 1975a) and consists of apparently equal amounts of two types of subunits, I and II (Carter and Etzler, 1975b). These subunits have similar amino acid and carbohydrate compositions (Carter and Etzler, 1975b), identical NH_2-terminal amino acid sequences (Etzler *et al*, 1977), and show reactions of identity when run in immunodiffusion against antisera made to either subunit or to the intact lectin (Carter and Etzler, 1975b). The NH_2-terminal halves of these subunits isolated after CNBr cleavage appear to be identical (Carter and Etzler, 1975c), but the subunits differ at their COOH-terminal ends (Carter and Etzler 1975b, c) and Subunit I is slightly larger than Subunit II (Carter and Etzler 1975b). The above information suggests that these two subunits may represent different degrees of completion or modification of a common polypeptide chain.

Equilibrium dialysis studies on the lectin showed that it has two carbohydrate binding sites per molecule (Etzler *et al*, 1981). Haemagglutination, quantitative precipitin, and affinity electrophoresis studies of the isolated subunits showed that only Subunit I has carbohydrate binding properties and that this subunit may be primarily responsible for the carbohydrate binding properties of the lectin (Etzler *et al*, 1981). This suggestion is supported by our recent finding that a monoclonal antibody that appears to be directed against a determinant on the lectin at or near the active site, reacts only with Subunit I (Borrebaeck and Etzler, 1978). The small difference in structure at the COOH-terminal ends of these subunits may thus be important for the activity of the lectin.

Distribution of the lectin

The distribution of the lectin during the life cycle of the plant was followed using a sensitive radioimmunoassay based upon the ability of lectin to inhibit the precipitation of radiolabelled lectin by antibodies to the seed lectin (Talbot and Etzler, 1978a). The lectin was not detected in the seeds during the first 26 days after flowering but appeared on day 27 and reached a maximum level by the 28th day (figure 1). High levels of lectin were maintained in the dry seed where the lectin accounts for about 9% of the total nitrogen of the seed extract (Etzler and Kabat, 1970; Talbot and Etzler, 1978a).

The lectin of germinating seeds is confined to the cotyledons (Talbot and Etzler, 1978a) where it appears to be associated with the storage granules or protein bodies (Etzler *et al.* unpublished). As the cotyledons are absorbed, the lectin disappears at about the same rate as the other cotyledon proteins (figure 2) (Talbot and Etzler, 1978a). Although no lectin was detected in the roots, low levels of lectin were found in the stems and leaves of the plant (Talbot and Etzler, 1978a). Attempts to isolate and characterize this lectin from the stems and leaves showed that it was not identical to the seed lectin but gave a precipitin band of only partial identity to the seed lectin when run in immunodiffusion against antisera to the seed lectin (Talbot and Etzler, 1978a, b). The properties of this cross reactive material are described below.

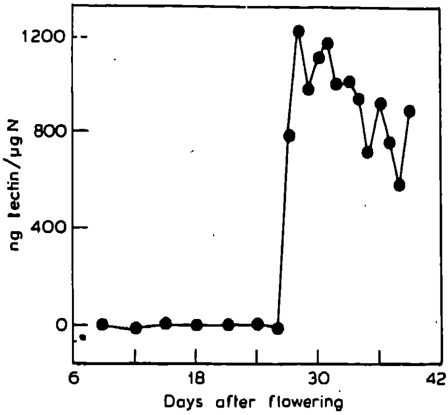

Figure 1. Lectin content of developing seeds of *D. biflorus* plant. At various times after flowering, the two to six seeds in a pod were pooled, homogenized, and assayed for lectin and nitrogen. Each point is the average of data from seeds of two to six pods except for days 29, 35, 36 and 37, when the seeds from only one pod were assayed. (Reprinted from Plant Physiology **61**, 847–850, Talbot and Etzler, 1978a, American Society of Plant.Physiologists).

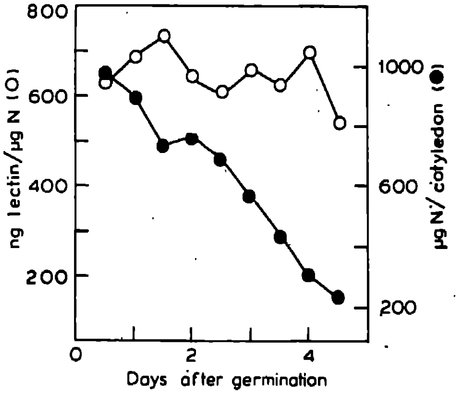

Figure 2. Lectin content of cotyledons during germination of seeds. At 12 h intervals from the time of planting, cotyledons were homogenized and assayed for nitrogen and lectin. (O), ng of lectin/μg of N; (●), total amount of N in cotyledon. Each point is the average of 12 assays. (Reprinted from Plant Physiology **61**, 847–850, Talbot and Etzler 1978a, American Society of Plant Physiologists).

Properties of 'lectin-like' glycoprotein from stems and leaves

The isolated cross reactive material from the stems and leaves of the *Dolichos biflorus* plant had a similar amino acid and carbohydrate composition to the seed lectin (Talbot and Etzler, 1978b). The cross reactive material is a dimer composed of a subunit identical in electrophoretic mobility to Subunit I of the seed lectin, and a subunit with a higher molecular weight than either of the seed lectin subunits (figure 3). Both the subunits of the cross reactive material have identical NH_2-

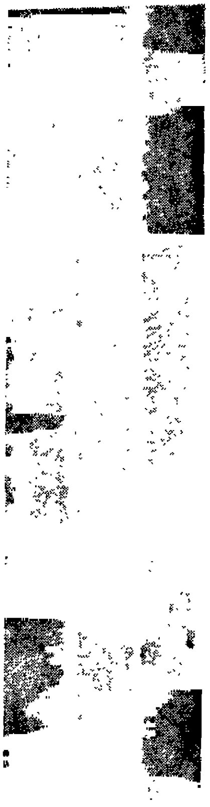

Figure 3. Discontinuous polyacrylamide gel electrophoresis of cross reactive material and seed lectin isolated from *Dolichos biflorus* and placed on pH 9.7 glycine gels in the presence of 0.1% soidum dodecyl sulphate and8.0M urea.The gel at left shows seed lectin subunits; the gel at right shows CRM subunits; the middle gel shows a mixture of CRM and seed lectin subunits. Gels are stained with Coomassie Brilliant Blue. (Reprinted from Phytopathology **71,** 744-746, Etzler, 1981).

terminal sequences (Talbot and Etzler, 1978b), and these sequences are identical to the seed lectin subunit sequences with the exception of an aspartate instead of an asparagine at the second residue (Etzler *et al.,* 1977; Talbot and Etzler, 1978b). This similarity of subunits indicates that the cross reactive material and lectin subunits may all be modifictions of a common polypeptide chain.

When assayed under the normal conditions for testing lectin activity, the cross reactive material did not agglutinate nor inhibit the agglutination of type A erythrocytes nor react with blood group A substance in solution (Talbot and Etzler 1978b). Lowering the ionic strength, however, enabled the cross reactive material to bind to affinity columns of blood group A substance (Etzler and Borrebaeck, 1980). The cross reactive material may therefore be considered as a lectin in its own right. Affinity electrophoresis studies indicated that the cross reactive material may have a somewhat broader carbohydrate specificity than the seed lectin (Etzler and Borrebaeck, 1980).

Distribution of 'lectin-like' glycoprotein

Immunofluorescence microscopy and cell fractionation studies indicate that a substantial portion of the cross reactive material is associated with the cell walls of the plant. This cross reactive material can be released from the cell walls by treatment with cellulase and pectinase (Etzler *et al.*, 1979).

Using a radioimmunoassay with antibodies specific for the cross reactive material, the distribution of soluble and cell wall associated cross reactive material was followed during early development of the plant (Roberts and Etzler, 1980). Only low levels of cross reactive material were detected during the first two days of germination, but on the third day the cross reactive material content began to increase and showed a 20-fold increase by the fifth day (Roberts and Etzler, 1980). This cross reactive material was localized in the epicotyl and leaves and was almost equally distributed between the soluble and particulate fractions. No cross reactive material was detected in the roots and only low amounts were found in the hypocotyl and cotyledons (Roberts and Etzler, 1980).

A fungal infection, classified as *Erysiphe pisi*, that occurred in our greenhouse caused an apparent elevation of cross reactive material levels in the leaves and stems of the plant,as detected by immunofluorescence microscopy. Other infections agents (*Botrytis cinerea* and *Pseudomonas phaseolicola*) and wounding the plants also produced a similar apparent elevation in the cross reactive material (Gibson and Etzler, 1979). These results must be considered as preliminary, however, since they could not be confirmed by radioimmunoassays of extracts of the infected or wounded tissues. Investigations are now in progress to determine whether this failure may be due to a degradation of the elevated cross reactive material during extraction.

Implications of these studies on the role(s) of lectins in plants

The localization of the *Dolichos biflorus* seed lectin in the cotyledons of the plant and its rate of disappearance relative to the other cotyledon proteins,suggests that this lectin may indeed play a rôle as a storage protein for use as food for the plant during germination as suggested many years ago by Boyd (1963). Seed lectins in some other plants have been found to have similar distributions (Lis and Sharon, 1981).

The failure to find lectin in the roots of the *Dolichos biflorus* plant does not support the hypothesis that lectins may be involved in the attraction of symbiotic *Rhizobia*. Some other investigators have also failed to find appreciable levels of lectins in roots, although other studies indicate their presence in the roots of some plants (Lis and Sharon, 1981).

The discovery of the cross reactive material in the stems and leaves of the *Dolichos biflorus* plant and its close structural relationship to the seed lectin, raise the possibility that this molecule may be the functional lectin in the plant and that the seed lectin may be its degradation product. The ability to activate or inactivate the cross reactive material depending upon the ionic strength of the solution suggests that the activity of this lectin may be under some type of regulatory control. The association of the cross reactive material, with the cell walls of the leaves and stems and its increase during the period of rapid growth, support the suggestion by Kauss and Glaser (1974) that lectins may be involved in cell wall elongation. The preliminary indications, that wounding or fungal infection may increase the level of cross reactive material, indicate that perhaps this molecule might also function in maintaining the integrity of the cell wall in response to stress (Etzler, 1981).

A cross reactive material to a seed lectin has also been reported in the leaves of *Phaseolus vulgaris* (Mialonier *et al.*, 1973) and further studies on the distribution of lectins in other plants may reveal similar 'lectin-like' substances in stems and leaves. Further studies of the distribution and properties of these molecules and their relationship to the seed lectin may aid in obtaining an eventual understanding of the role(s) of lectins in plants.

Conclusions

A study of the distribution of the *Dolichos biflorus* lectin during the life cycle of the plant showed that the seed lectin appears at about 27 days after flowering and is concentrated in the cotyledons. Although this lectin disappears during the absorption of the cotyledons, a related molecule that cross reacts with antibodies to the seed lectin begins to accumulate in the stems and leaves of the plant. This cross reactive material appears to have one subunit in common with the seed lectin and another subunit that has a higher molecular weight than the seed lectin subunits. Both the cross reactive material and seed lectin subunits may represent different degrees of completion or modification of a common polypeptide chain.

The carbohydrate binding activity of the cross reactive material can be altered by the ionic strength of the solution and a substantial portion of this 'lectin-like' glycoprotein appears to be associated with the cell wall. There are preliminary indications that the level of this molecule may be elevated upon wounding or fungal infection of the plant.

Further studies on the distribution and properties of lectins during the life cycle of the plant may be valuable in obtaining a better insight into the physiological role(s) of these molecules in the plants.

Acknowledgements

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Metal ion binding to concanavalin A

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Abstract. Metal ion activation of saccharide binding has been studied for concanavalin A near pH 7.0. Although two metal ions, a transition metal ion and a Ca^{2+} ion, can bind, both are not required. Ca^{2+} alone, Mn^{2+} alone, or Ca^{2+} with other transition metal ions can activate this lectin. Only one Ca^{2+} ion per subunit or only one Mn^{2+} per subunit is sufficient. Metal ion binding was studied by magnetic resonance techniques and direct binding assays. Saccharide binding activity was monitored by following the fluorescence of 4-methylumbelliferyl α-D-mannopyranoside. When Ca^{2+} binds to demetalized concanavalin A, the transition metal ion site is hindered. When Mn^{2+} alone binds to demetalized concanavalin A, saccharide binding activity is induced. A subsequent conformational change, not necessary for carbohydrate binding activity, covers the Mn^{2+}.

Keywords. Concanavalin A; metal ions; lectin; saccharide binding.

Introduction

Concanavalin A, the most extensively studied of all lectins, possesses two metal ion binding sites per 35,500-dalton subunit. Although the metal ions are required for activity, the ions do not coordinate with atoms of the sugars which are bound by concanavalin A (Con A) (Brewer *et al.*, 1973a, b; Alter and Magnuson, 1974; Villafranca and Viola; 1974; Becker *et al.*, 1976; Hardman and Ainsworth, 1976). Each subunit has one sugar binding site and one binding site for a transition metal ion designated *S*1, ion and one site for a Ca^{2+} ion, designated *S*2 (Shoham *et al.*, 1973). A variety of transition metal ions bind to *S*1 but requirements at *S*2 are more rigid with only a limited number of ions such as Cd^{2+} being able to replace Ca^{2+}.

In our initial work we were interested in repeating studies on the metal ion requirements for sugar binding activity. In control experiments near pH 7.0 where Con A is tetrameric, we noticed that when Ca^{2+} alone was added to demetallized Con A, glycogen agglutination would occur. From this point we were forced to question the standard idea that first a transition metal ion binds to Con A with subsequent formation of the *S*2 site. This results in formation or activation of the sugar binding site. This hypothesis is based primarily on work done near pH 5.0 where Con A is dimeric.

Abbreviations used: Con A, Concanavalin A; MUM, 4-methylumbelliferyl α-D-mannopyranoside; Mops, morpholinopropanesulphonate.

Dean and Homer (1973) reported that the fluorescence of 4-methylumbelliferyl α-D-mannopyranoside (MUM) was completely quenched upon binding to Con A. This provided a very sensitive assay for studying the activity of Con A containing a variety of metal ions. The fluorescence properties of this compound have also been used extensively to study kinetics of saccharide binding to Con A (Clegg *et al.*, 1981). In the work reported below we have utilized the quenching of the umbelliferyl derivative as a tool for measuring how many and what kind of metal ions are required for conferring sugar binding activity on demetalized Con A.

Materials and methods

Concanavalin A

Concanavalin A was isolated from jack bean meal by affinity chromatography as described by Agrawal and Goldstein (1967). Protein concentrations were determined spectrophotometrically at pH 6.4 to 7.0 by using an absorbance $A^{1\%,\ 1\,\mathrm{cm}}_{280\,\mathrm{nm}} = 13.7$ (Yariv *et al.*, 1968). Demetalized Con A (or apo-Con A) was prepared by dialysis against 0.1 N HCl. Metal ion contamination was monitored using atomic absorption spectroscopy. Removal of transition metal ions was relatively simple, but plastic glassware (usually polypropylene) was used to keep Ca^{2+} ion contamination to a minimum. Ca^{2+} contamination was always checked before and after experiments and only experiments where Ca^{2+} contamination was less than 5% on a molar basis with Con A subunits are reported.

Fluorescence measurements

Fluorescence measurements were carried out on 2-ml samples containing protein, MUM, and the appropriate metal ion or ions. The cuvette holder of the Perkin-Elmer MPF-3L fluorescence spectrophotometer was thermostated at the appropriate temperature. Fluorescence was monitored with an excitation wavelength of 350 nm and an emission wavelength of 375 nm. Protein concentrations were typically 0.1 mM in Con A subunits and MUM concentrations ranged between 0.02 and 0.2 mM.

Equilibrium dialysis

Three different methods were used for these experiments. Polycarbonate cells with approximately 2-ml volume on each side of the dialysis membrane were filled with a total of 1.0 ml of the appropriate Con A solution on one side and 1.0 ml of buffer on the other. Metal ions were added to appropriate concentrations. The whole cell was agitated slowly at the desired temperature. Other dialysis experiments were carried out in polypropylene test tubes. Con A solutions in small dialysis tubing were placed into the test tubes and the tubing was covered with appropriate buffer. Test tubes were agitated slowly at the desired temperature. In some cases, dialysis bags with Con A were placed in Erlenmeyer flasks and the dialysate was changed many times with buffer containing metal ion of the desired free concentration. In these experiments, free metal ion concentrations were determined by the choice of buffer. For example, dialysis against repeated changes of buffer containing 10 μM Mn^{2+} gave a free Mn^{2+} concentration of 10 μM. In other cases metal ion concentrations were monitored with radioactive nuclides or atomic absorption spectrophotometry.

Magnetic resonance experiments

Electron spin resonance spectra of Mn^{2+} were obtained on a Varian E-3 or E-9 ESR spectrometer. In following Mn^{2+} binding to Con A, the low field extremum of the Mn^{2+} signal was observed as a function of time.

Nuclear magnetic resonance experiments were carried out on a Bruker WH-90/SXP NMR spectrometer, or Varian A-60 NMR spectrometer. Spin-lattice relaxation times T_1 of water protons were determined at 20.5 MHz. T_2 values were determined from water proton line widths.

Results

In early experiments in our laboratory using demetalized Con A, we tested for activity by glycogen precipitation. Total activity was determined either by measuring the amount of protein in the Con A-glycogen precipitate or by observing scattered light in a spectrophotometer. As the Con A-glycogen precipitate increased, the light scattered would increase. In these experiments metal ion requirements were tested by adding metal ions to demetalized Con A in solution with glycogen. Our experiments showed that a precipitate not only formed when a transition metal ion and Ca^{2+} were added, but that glycogen precipitates formed near pH 7.0 when Ca^{2+} was the only divalent metal ion present. The experiments presented below were designed to re-examine the requirements for transition metal ions and Ca^{2+} ions.

The x-ray studies identifying the metal sites (Hardman, 1973; Becker *et al*, 1975) suggests that if Ca^{2+} alone activates Con A, Ca^{2+} should inhibit transition metal ion binding. The time course of Mn^{2+} binding to demetallized Con A in the presence of various amounts of Ca^{2+} was examined by following the amplitude of the Mn^{2+} ESR signal. The signal of free Mn^{2+} in solution is approximately 15 times that of an equivalent amount of Mn^{2+} bound to Con A. The amplitude of the ESR signal observed is a combination of both bound and free Mn^{2+}. The bound Mn^{2+} contributes so little to the intensity at a given point that the intensity measured is a good measure of the free Mn^{2+}. If $Mn^{2+}$is added to demetallized Con A, the resonance of free Mn^{2+} decreases with time as Mn^{2+} binds to the protein. As shown in figure 1, the amount of free Mn^{2+} rapidly decreases to a relatively low concentration when Mn^{2+} is added to demetallized Con A. When Ca^{2+} is present before Mn^{2+} is added, the rate of disappearance of Mn^{2+} is markedly reduced.

When Mn^{2+} binds to demetalized Con A, the relaxation rate of the solvent water protons are greatly enhanced. This can be observed in proton magnetic resonance experiments examining either T_1, the spin-lattice, or T_2, the spin-spin relaxation time. Early studies by Carver and Barber (1973) and Brown *et al.* (1977), among others, used water relaxation to study metal ion complexation to Con A. We found (figure 2) that the water proton T_2 relaxation enhancement ε^* (Dwek, 1973) observed in the presence of Mn^{2+} bound to demetallized Con A decreased slowly with time and more slowly than the disappearance of Mn^{2+} (figure 1). The enhancement value decreased slowly over a period of 1 to 2 h when no Ca^{2+} was present. In the presence of Ca^{2+} (figure 2), values decreased in a time course almost identical to the disappearance of Mn^{2+} in the presence of Ca^{2+} (figure 1). If

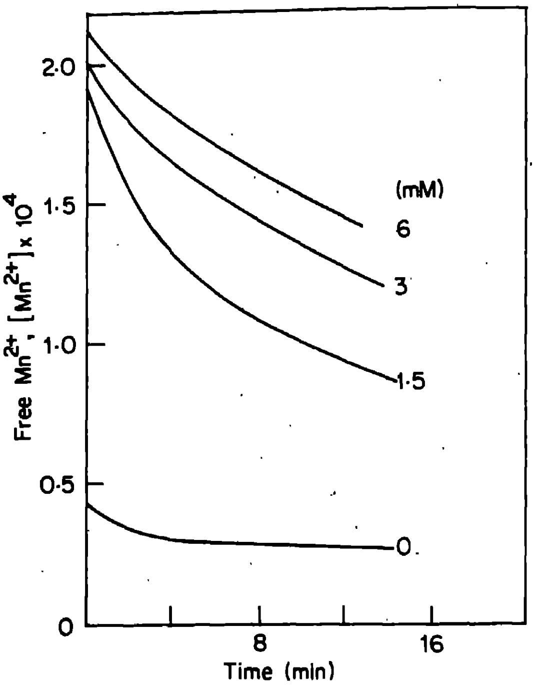

Figure 1. Amplitude of the lowfield extremum of the Mn^{2+} ESR signal as a function of time. At time zero 0.34 mM, Con A monomers were incubated with 0.32 mM, Mn^{2+} and the amount of Ca^{2+} given on the figure. The buffer was 0.05 M Mops, pH 6.5, 0.2 M NaCl; temperature was 18° C.

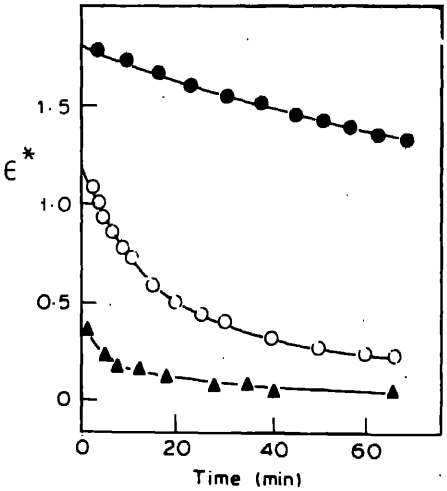

Figure 2. Enhancement for T_2 relaxation rates following addition of Mn^{2+} to Con A. Individual points were calculated from line widths at times given. The Con A subunits were 0.33 mM; Mn^{2+} was added to a total concentration of 0.26 mM. Mn^{2+} was added to demetallized Con A, but no Ca^{2+} present (●). Mn^{2+} added to demetallized Con A, preincubated with 2.8 mM Ca^{2+} for 30 min, (○). Ca^{2+}, 2.8 mM, added to Con A incubated for 80 min with 0.26 mM Mn^{2+}, (▲). Buffer was 0.05 Mops, pH 6.45, 0.2 M NaCl; temperature was 28.5° C.

Ca^{2+} were added to Con A containing only Mn^{2+}, a rapid decrease in ε^*, the relaxation rate enhancement, would occur. Ca^{2+} binding to Con A, therefore, produced two effects. One is detected in the ESR experiment and the other is detected in the water proton magnetic resonance experiment. Ca^{2+} inhibits the rate at which Mn^{2+} binds to Con A, but Ca^{2+} also will promote a conformational change which covers the bound Mn^{2+} ion so that interaction of water with the bound Mn^{2+} is blocked, at least as detected in the NMR experiment.

Water proton interaction was also studied by examining the time dependence of the spin lattice relaxation time T_1. Brown *et al.* (1977) had demonstrated an interesting concentration effect when Mn^{2+} was added to demetallized Con A. At low stoichiometric ratios of Mn^{2+} to Con A, the enhancement in relaxation rates was observed to first increase and then decrease as the ratio increased. We repeated this experiment at 23° C with longer-times for the Mn^{2+}–Con A interactions to reach equilibrium, but also noted that at 5° C with little delay after mixing metal ion and protein no marked reduction in relaxation enhancement occurred (figure 3), unless the T_1 measurement were made after very long incubation periods. The T_1 experiment is detecting two species. One is a Con A conformation with Mn^{2+} open to solvent water. This conformation changes to one where water is not as freely exchanging with waters of hydration on the Mn^{2+}. We were able to measure the changes in T_1 as a function of time which really monitored the change from the open to the closed conformation. Standard kinetic analyses (Daniels *et al*, 1962) presented in figure 4 shows that the conversion is a simple first order process. The activation energy for this process which occurs even when Ca^{2+} is not present was 22.3 kcal mol^{-1}.

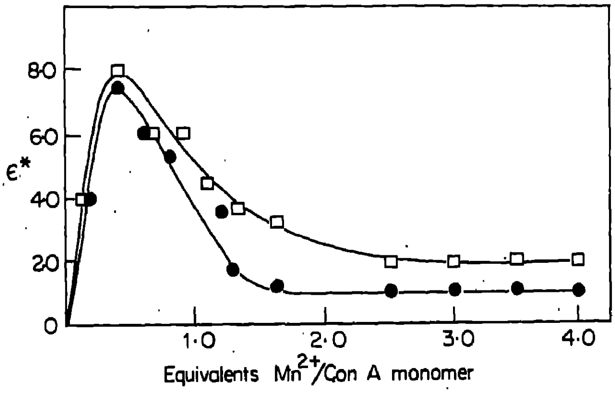

Figure 3. Enhancement for T_1 relaxation rates following addition of Mn^{2+} at various levels to demetallized Con A. The buffer was 0.05 M Mops, pH 6.5, 0.9 M KCl (the same results were obtained in acetate buffered at 6.5 to reproduce other reported values, Brown *et al*, 1977). Sample prepared at 5°C, (□); sample prepared at 23°, (●). Note that the kinetic analysis in figure 4 is following conversion from (□) to (●) at a Mn^{2+}/Con A ratio of 3.0.

The above experiments show that Con A can bind Ca^{2+}, near pH 7.0, without first binding a transition metal ion. When a transition metal ion such as Mn^{2+} binds, there is a slow conformational change which covers the Mn^{2+} ion. Ca^{2+} bound to demetalized Con A inhibits the rate at which Mn^{2+} binds but, when pre-

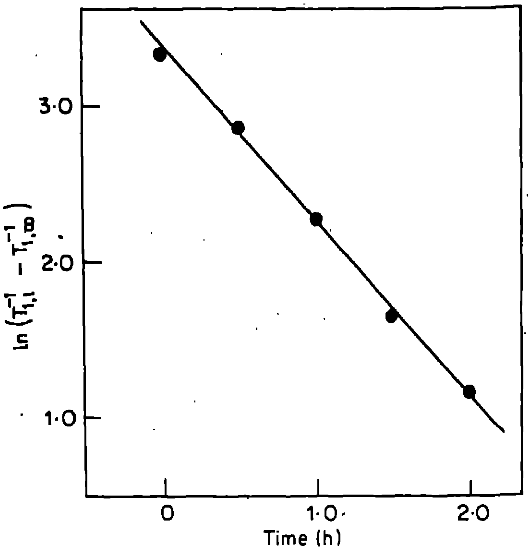

Figure 4. Kinetic analysis of conversion of unstable Con A to stable Con A. Experiment carried out by measuring T_1 relaxation times following Mn^{2+} addition. Mn^{2+} was added at 0–5°C and solution was warmed to 32°C. $T_{1,t}$ is the observed T_1 at various times (in h) and $T_{1,\infty}$ is the spin-lattice relaxation time at very long time periods when no further change with time is detected. The first-order rate constant is 0.96 h^{-1}.

sent, Ca^{2+} increases the rate of the conformational change covering the bound Mn^{2+}. The following experiments were designed to determine which metalized states have saccharide binding activity.

The fluorescence of 4-methylumbelliferyl α-D-mannopyranoside was used to monitor saccharide binding activity. Activity was generally determined through Scatchard analyses (Scatchard, 1949) in order to demonstrate the extent of active Con A in addition to the binding affinity. To demetalized Con A was added the appropriate amount of metal ion. Binding analyses using the quenching of the MUM fluorescence could then be carried out by titrating the Con A with MUM in the fluorescence cuvette. Results for Con A with only Ca^{2+} and for Con A with only Mn^{2+} are shown in figure 5. These data show that the amount of active Con A is approximately equal to the total amount of divalent ion supplied. For example, when the bound metal ion to Con A monomer ratio is 0.5 the amount of active Con A was about 50%. The slopes of the plots were the same for different concentrations of a given metal ion, although the abscissa intercept demonstrated that all the Con A was not activated. The similarity of the slopes showed that no intermediate binding states were produced as all active species had the same affinity for MUM. In these studies Zn^{2+}, Ni^{2+} and Co^{2+} alone could not activate Con A for saccharide binding, although Scatchard analyses of metal binding using radioactive nuclides showed that one, and only one, of these ions or Mn^{2+} bound per Con A monomer. In addition, Scatchard plots for binding of these four ions did not deviate from linearity, suggesting that only one type of binding site is present.

Thus far our results have shown that Ca^{2+} and a transition metal, Ca^{2+} alone, or Mn^{2+} alone could induce the saccharide binding activity. Distance measurements between Mn^{2+} and the bound saccharide were made on the two forms to obtain infor-

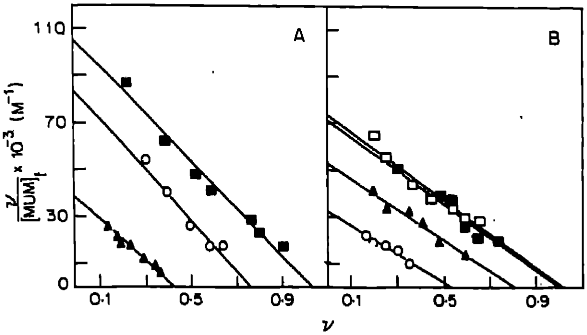

Figure 5. Scatchard analysis of MUM binding activity for various metal additions to demetallized Con A. Panel A: Ca^{2+} only added to demetallized Con A. The ratio of Ca^{2+} to Con A monomers is 0.58, (▲); 0.87 (○), < 1, (■). Experiment carried out at pH 7.2, 5° C. Panel B: Mn^{2+} only added to demetallized Con A at Mn^{2+} to Con A ratios of 1.0 (○); 2.0, (▲); 5.0, (□); 10, (■). The ratio of Mn^{2+} bound to Con A subunits at these values are 0.5, 0.8, 1.0, and 1.0, respectively (Christie *et al*, 1979). Experiment carried out at pH 6.5, 5° C.

mation about the location of the Mn^{2+} ion. Distance measurements between Mn^{2+} and the fluorine on N-trifluoroacetyl-D-glucosamine were made in NMR experiments similar to those reported previously (Alter and Magnuson, 1974). Fluorine T_1 and T_2 values were measured at 84.7 and 254.0 MHz. T_1's were measured by inversion-recovery and T_2's were calculated from line widths. Con A containing both Mn^{2+} and Ca^{2+} and Con A with just Mn^{2+} were examined. The Mn^{2+}-F distances were found to be identical (Pandolfino *et al*, 1980), suggesting that Mn^{2+} in each form is the same site.

The water proton relaxation experiments clearly demonstrated that when Mn^{2+} alone is bound to Con A, two conformations can exist. The initial one slowly converts to the more stable form. At 5° C, the conversion takes several days. Binding of MUM by both conformers was examined. Both species bound MUM with the same affinity (Christie *et al*, 1980). In addition, no time dependence for induction of saccharide binding activity was detected. These results have shown that Mn^{2+} binding at pH values near 7 is enough to activate the sugar binding site. The conformational change, subsequent to Mn^{2+} binding and studied so extensively by various workers, is not necessary for activity. With Ca^{2+} activated Con A in the absence of a transition metal ion, the Con A appears to have a similar conformation to Con A with two metal ions, as the affinities for saccharides are essentially identical.

Discussion

A model which accounts for metal ion binding data is presented in figure 6. Different states are represented by symbols and the metal ions within each symbol are used to indicate the binding to each subunit. All the circles represent monomers with saccharide binding activity. We suggest that it is necessary for the Ca^{2+} only species to lose Ca^{2+} before being able to bind a transition metal ion. The intermediate state could be the demetalized triangle or another unidentified conformation. Mn^{2+} alone must produce a different conformation, a circle, than other transition metal ions, a hexagon, but Ca^{2+} can convert the latter to the active species.

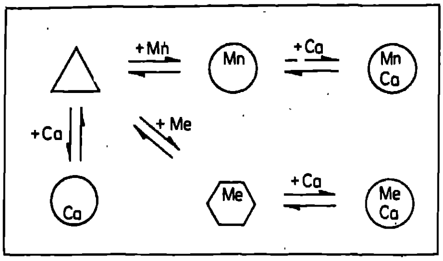

Figure 6. Model for metal binding to each subunit of Con A. Conformations when metalized have metal within symbol. Me represents transition metal ion not Mn^{2+}. Circles are active, saccharide binding conformations.

The two metal sites, *S*1 for the transition metal ion and *S*2 for the Ca^{2+} ion, do not both need to be occupied for activity near pH 7.0. One Mn^{2+} is sufficient with distance measurements showing that the ion is most likely in *S*1. We suggest that Ca^{2+} binds at *S*2 with or without the presence of a transition metal ion. We have no direct determination for this, but difference ultra-violet (UV) spectra for Con A with only Ca^{2+} versus Con A with Ca^{2+} and Mn^{2+} ion are essentially identical (Munske and Magnuson, unpublished observation). The saccharide binding affinity also appears to be identical for Con A with both Mn^{2+} and Ca^{2+} or with just Ca^{2+}.

In summary, demetalized Con A can bind either a transition metal or a Ca^{2+} ion. Activation of the sugar binding site probably occurs simultaneously with Mn^{2+} binding and a slow conformational change, unrelated to the sugar binding site, follows and covers the Mn^{2+} site. Only one Mn^{2+} binds per subunit (Christie *et al*, 1979). When Ca^{2+} binds first, sugar binding activity is induced and the Ca^{2+}, as would be expected from crystallographic results, prevents Mn^{2+} from binding. The Ca^{2+} most probably must dissociate before Mn^{2+} can bind and the Ca^{2+} which is present then enhances the rate at which the Mn^{2+} is covered by other ligands. These steps, it should be noted, have led to much confusion in previous interpretations. The covering of Mn^{2+} by movement of a protein segment was suggested early by Barber and Carver (1973).

Early work on Con A assumed that Ca^{2+} could only bind after the transition metal ion had bound to *S*1 and induced the formation of the Ca^{2+} site *S*2. Kinetic studies of transition metal binding in the presence of Ca^{2+} are confusing because Ca^{2+} markedly reduces binding rates. Indeed, care must be taken to prevent Ca^{2+} contamination.

In some cases MUM fluorescence has been used to measure the induction of saccharide binding activity. The technique is very sensitive and it is possible to measure easily the activity of a small percentage of the Con A present. For example, when Co^{2+} is added to demetallized Con A, a 5% contamination with Ca^{2+} would generate 5% active Con A. If Co^{2+} is added in large excess, the Co^{2+} solutions can contain relatively large amounts of Ca^{2+} which could activate a relatively large portion of the Con A. In all experiments it is necessary, we feel, to determine the relative amounts of active Con A.

The work presented here demonstrates that careful studies of metal ion requirements for Con A, and other lectins, must include two points. Metal ion contamination, especially by Ca^{2+}, must be checked, before and after experimentation, by using atomic absorption spectrophotometry or other suitable technique. Extent of activity must be checked. We prefer Scatchard analyses using the fluorescence of MUM but other quantitative methods may be necessary with other lectins.

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Carbohydrate mediated recognition of lysosomal enzymes by cell surface receptors

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Abstract. The recognition of lysosomal enzymes by various carbohydrate specific cell surface receptors is reviewed. In particular the biosynthesis of mannose 6-phosphate residues in lysosomal enzymes and their role for targeting of lysosomal enzymes to lysosomes are discussed.

Keywords. Mannose 6-phosphate; carbohydrate specific receptors; lysosomal enzyme transport.

Most of the lysosomal enzymes that have been assayed for are glycoproteins (Strawser and Touster, 1980). Among the physiological functions ascribed to the carbohydrate moiety in lysosomal enzymes, were a possible contribution to the catalytic activity and stability towards lysosomal proteases. At present no example is kown in which the catalytic activity of a lysosomal enzyme was shown to depend on its carbohydrate moiety. In contrast, removal or modification of the carbohydrate, had no effect on the catalytic properties of the lysosomal enzymes so far studied. Lysosomal enzymes display a considerable stability towards various proteinases. It is, however, not known to what extent the carbohydrate moiety contributes to the stability of lysosomal enzymes in their physiological environment. Several examples of non lysosomal glycoproteins are known in which oligosaccharides seem to function in stabilizing the polypeptides (Gibson *et al*, 1980).

In the past ten years it became evident that the carbohydrate moiety in lysosomal enzymes is of importance for the transfer of lysosomal enzymes into lysosomes. A variety of cells expose on their surface receptors that recognize the carbohydrate part of lysosomal enzyme and transfer the receptor bound lysosomal enzymes into lysosomes or related structures. Most of the receptors have been isolated and characterized (Neufeld and Ashwell, 1980).

In table 1 the carbohydrate specific receptors mediating endocytosis of lysosomal enzymes are listed up along with the cell types where they have been identified. The receptors specific for galactose/N-acetylgalactosamine, mannose/N-acetylglucosamine or L-fucose residues in glycoproteins are not specific for lysosomal enzymes, and it is not clear at present whether they mediate the transfer of lysosomal enzymes under normal conditions.

The recognition of lysosomal enzyme *via* mannose 6-phosphate specific receptors has gained special interest. Hickman and Neufeld (1972) had shown that genetic deficiency of a recognition marker in lysosomal enzymes from I-cell (mucolipidosis II) fibroblasts is accompanied by loss of newly synthesized lysosomal enzymes into the medium and intracellular deficiency of thse enzymes (II).

Table 1. Carbohydrate specificity of cell surface receptors for lysosomal enzymes

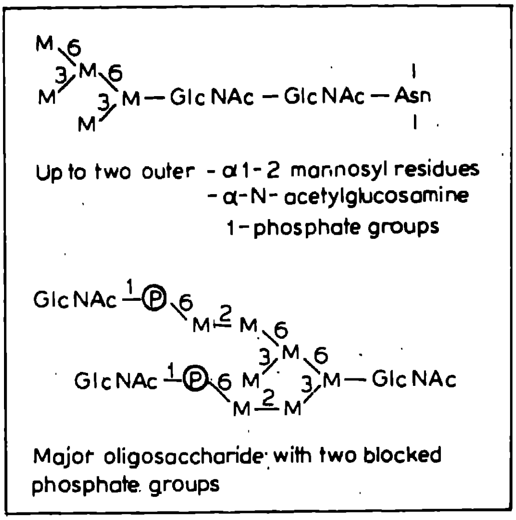
Carbohydrate recognized	Receptors expressed in
Mannose 6-phosphate	Fibroblasts (Neufeld and Ashwell, 1980) Smooth muscle cells (Hasilik *et al.*, 1981) Hepatocytes (Ullrich *et al.*, 1979a)
Mannose/N-acetylglucosamine	Macrophages (Stahl *et al.*, 1978; Ullrich *et al.*, 1979b) Hepatocytes (Ullrich *et al.*, 1979a)
Galactose/N-acetylgalactosamine	Hepatocytes (Ullrich *et al.*, 1979a; Furbish *et al.*, 1978)
L-Fucose	Hepatocytes (Furbish *et al.*, 1980) Macrophages (Shepherd *et al.*, 1981)

This indicated that the physiological function of the recognition marker in lysosomal enzyme, which was later identified as that containing mannose 6-phosphate residues (Kaplan *et al.*, 1977; Sando and Neufeld, 1977; Ullrich *et al.*, 1978; von Figura and Klein, 1979; Distler *et al.*, 1979; Natowicz *et al.*, 1979; von Figura *et al.*, 1979;Hasilik and Neufeld,1980)may be that of a signal,allowing the transfer of newly synthesized lysosomal enzymes into lysosomes. Further evidence for the requirement of a phosphorylated recognition marker for the proper transfer of lysosomal enzymes was provided by experiments with tunicamycin (Varki and Kornfeld, 1980; von Figura *et al.*, 1981). Tunicamycin interferes with the formation of asparagine linked oligosaccharides, including the mannose 6-phosphate containing ones. The lysosomal enzymes formed in the presence of tunicamycin which are smaller by size are mainly secreted. Intracellular enzyme activities decrease and lysosomal enzymes bound to receptors at the cell surface are absent as in I-cell fibroblasts.

If the function of mannose 6-phosphate containing recognition marker is that of a signal for the segregation of lysosomal enzymes from other glycoproteins and their transport into lysosomes, the recognition signal should be specific for lysosomal enzymes (and non-enzymic lysosomal glycoproteins), and operate in many cell types. So far lysosomal enzymes (von Figura and Klein, 1979; Distler *et al.*, 1979; Natowicz *et al.*, 1979; Hasilik and Neufeld, 1980; Varki and Kornfeld 1980; Hasilik *et al.*, 1980) and their equivalent in yeast, the vacuolar enzymes (Hashimoto *et al.*, 1981) are the only class of glycoproteins in which mannose 6-phosphate containing structures have been detected. Mannose 6-phosphate specific receptors are present at the cell surface and intracellular membranes (Gonzalez-Noriega *et al.*, 1980; Fischer *et al.*, 1980a). They have been found in every organ thus far examined (Fischer *et al.*, 1980b) and cell surface associated and intracellular receptors may operate at two different levels. Mannose 6-phosphate specific receptors at the cell surface transfer lysosomal enzymes form the extracellular compartment into lysosomes. This pathway operates in fibroblasts, hepatocytes, smooth muscle cells and glial cells, whereas endothelial cells and chondrocytes are not capable of internalizing lysosomal enzymes *via* endocytosis (von Figura *et al.*, 1980). The uptake of lysosomal enzymes from the extracellular compartment seems to be of minor importance for the equipment of the cell with lysosomal enzymes. Selective inhibition of the endocytotic pathway has practically no effect on cellular levels of lysosomal enzymes (von Figura and Weber, 1978; Sly and Stahl, 1978; Vladutiu, and Rattazzi, 1979). Of major importance for the transfer of newly synthesized lysosomal enzymes into lysosomes, seems their binding to intracellular (Golgi associated) mannose 6-phosphate specific receptors. It has been postulated that receptor bound lysosomal enzymes are either segregated from other glycoproteins into specific vesciles (primary lysosomes) which fuse with secondary substrate containing lysosomes (intracellular transfer) (Sly and Stahl, 1978), or that the receptor-enzyme complex is not segregated from other glycoproteins at an intracellular level, but is transported with secretory glycoproteins to the cell surface, where they remain largely bound to the cell surface and enter lysosomes only after endocytosis (transfer *via* cell surface) (von Figura and Weber, 1978). Both pathways do not exclude each other, and have so far not been discriminated experimentally.

Recently some progress was made in elucidating the pathway by which mannose 6-phosphate containing recognition markers are synthesized. High-mannose oligosaccharides in lysosomal enzymes are phosphorylate by transfer of N-acetylglucosamine 1-phosphate to C-6 hydroxyl of mannose residues.UDP-N-acetylglucosamine serves as donor for the N-acetylglucosamine 1-phosphate (Hasilik *et al*,1981; Reitman *et al*,1981). The enzyme is located in membranes fractionating with the Golgi marker galactosyltransferase (Waheed *et al*, 1981). Up to two N-acetylglucosamine 1-phosphate residues appear to become transferred per oligosaccharide. It is unknown whether a single enzyme is responsible for the transfer of both N-acetylglucosamine 1-phosphate residues. The transferase has been solubilized and partially purified from rat liver Golgi membranes. The solubilized transferase requires Mg^{2+} and transfer N-acetylglucosamine 1-phosphate only onto lysosomal enzymes, such as β-hexosaminidase and arylsulphatase *A* from various human and non-human sources, whereas glycoproteins such as ovalbumin, horse radish peroxidase and thyroglobulin did not serve as acceptors (Waheed. *et al*, 1981 unpublished). The deficiency of the transferase in I-cell fibroblasts explains the formation of lysosomal enzymes that lack the mannose 6-phosphate containing recognition marker (Hasilik *et al*, 1981). Fibroblasts from patients with mucolipidosis III, a disease closely related to I-cell disease, show residual activities of the transferase of less than 10% of controls (Reitman *et al*, 1981; Waheed, A., Hasilik, A., Cantz, M. and von Figura K., unpublished).

The product of the transferase are lysosomal enzymes with phosphodiester groups, that have been termed "blocked" phosphate groups, because of their inaccessibility to alkaline phosphatase. The blocking N-acetylglucosamine residues are removed by an α-N-acetylglucosaminyl phosphodiesterase, which is most enriched in the Golgi apparatus, but also present in other membranes of the smooth and rough endoplasmic reticulum (Varki and Kornfeld, 1980; Waheed *et al*, 1981a, b). The phosphodiesterase is distinguishable by several criteria from the lysosomal α-N-acetylglucosaminidase, which also cleaves the blocking N-acetylglucosamine groups (Varki and Kornfeld, 1980; Waheed *et al*, 1981). Apparently part of the blocking N-acetylglucosamine residues escapes from the action of the phosphodiesterase (Varki and Kornfeld, 1980; Hasilik *et al*, 1980; Tabas and Kornfeld, 1980). In β-hexosaminidase and cathepsin D, secreted by human skin fibroblasts, about 80% of the phosphate residues in high-mannose oligosaccharides are blocked (Hasilik *et al*, 1980). The structural features of the



Up to two outer - α1-2 mannosyl residues - α-N- acetylglucosamine 1-phosphate groups

Major oligosaccharide with two blocked phosphate groups

Table 2. Biosynthetic Intermediates of phosphorylated recognition markers in β-hexozaminidase and cathepsin D.

various oligosaccharides with blocked phosphate groups found in β-hexosaminidase and cathepsin D are summarized in table 2. In β-glucuronidase, and total glycoproteins, from mouse lymphoma cells, more than two dozens of different phosphorylated high-mannose oligosaccharides with either blocked, or unblocked phosphate groups, or both, were found (Varki and Kornfeld, 1980).

The elucidation of the pathways for the formation of the phosphorylated recognition marker allowed the identification of two new reactions involved in the processing of the carbohydrate in glycoproteins and of the primary defect in two genetic disorders. Among the interesting questions that came up by these studies are: What kind of signal in lysosomal enzymes directs the N-acetylglucosamine 1-phosphate transferase and confers to the required specificity of the acceptor in that reaction ? Which of the various phosphorylated oligosaccharides in lysosomal enzymes mediate binding to receptors and transfer into lysosomes ?

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Human erythrocyte specific lectin from the seeds of Indian coral tree, *Erythrina variegata* Linn, var. *orientalis* Linn, Merrill

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Abstract. Human erythrocyte specific lectin was isolated from the seeds of *Erythrina variegata* Linn. var. *orientalis* Linn. Merrill. The lectin preferentially agglutinated erythrocytes in the sequence of O>B>A = AB. The lectin was purified 19-fold by affinity chromatography on acid treated sepharose 4B with an yield of 81%. The purified lectin was found homogeneous on polyacrylamide gel electrophoresis. The erythroagglutination reaction was inhibited by N-acetyl-D-galactosamine, D-galactose and lactose at very low concentration. The haemagglutination by the purified lectin was not inhibited by different hexose and pentose sugars even at high concentration. The purified lectin was a glycoprotein and agglutinated leucocytes at 3 μg protein concentration. The lectin induced transformation of peripheral blood lymphocytes in cultures.

Keywords. *Erythrina variegata*; lectin purification; human erythrocyte specific; mitogen.

Introduction

It has been known for many years that extracts of a variety plant seeds contain haemagglutinins which are now called lectins. Plant lectins are carbohydrate binding proteins which recognize and interact with specific monosaccharide or oligosaccharide units and they agglutinate cells and/or precipitate glycoconjugates (Goldstein *et al*, 1980). Lectins are used extensively in immunohaematology as blood typing reagents and in testing erythrocyte polyagglutinable states (Prokop and Uhlenbruck, 1969; Levine, 1974; Gold and Balding, 1975; Brown and Hunt, 1978; Judd, 1979). They have proven to be useful tools for studying cell surface architecture and related membrane functions.The effect of plant lectins on eukaryotic cells is quite varied. Some are toxic, others are mitogenic for cells such as lymphocytes, and still others bind to the cell surface and apparently do not perturb cellular metabolism (Rapin and Burger, 1974; Nicolson, 1974; Lis and Sharon, 1977; Goldstein and Hayes, 1978; Hart, 1980; Weir, 1980).

Erythrina Linn (Leguminosae), a genus of trees or shrubs, rarely herbs, is widely distributed in tropical and subtropical regions. About eight indigenous species and ten introduced ones occur in India (The wealth of India, 1952). Within the genus *Erythrina*, erythroagglutinating activities of various specificities in crude extracts of the seeds had been demonstrated (Boyd, 1950; Cazal and Lalaurie, 1952; Tiggel-

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Abbreviation used: PBS, Phosphate buffered saline.

man-van Krugten *et al.*, 1956; Makela, 1957; Boyd *et al.*, 1961; Martin and Bomchill, 1966; Bird and Wingham, 1973; Lee *et al.*, 1977).

The present work describes a new human erythrocyte specific lectin, a glycoprotein, isolated from the seeds of the Indian coral tree *Erythrina variegata* (Linn.) var. *Orientalis* (Linn.) Merrill and some of its properties.

Materials and methods

Dry seeds, after the removal of the testa, were finely ground in a hand operated grinder avoiding generation of heat. The following steps were carried out at 4–6° C. Finely ground beans (10 g) were stirred overnight in 500 ml of 0.15 *M* phosphate buffered saline pH 7.2 (PBS). The resulting mixture was filtered through cheese cloth and centrifuged at 20,000*g* for 60 min to remove the seed debris. The supernatant (crude extract) was subjected to $(NH_4)_2SO_4$ fractionation of proteins. The precipitates obtained at different $(NH_4)_2SO_4$ saturations were dissolved in PBS contaning 0.1 mM each of $CaCl_2$, $MnCl_2$ and $MgCl_2$, and dialysed extensively against the same buffer. An aliquot (100 mg protein) of the most active fraction 30–70% $(NH_4)_2SO_4$ saturation was applied to a column of acid treated sepharose 4B (Ersson *et al.*, 1973) through washing of the column (30 × 1.2 cm) with PBS containing metal ions and elution of the lectin from it with 0.05 *M* glycine HCl buffer, pH 3.0 in 0.5 *M* NaCl. The active fractions were pooled together, neutralized by saturated solution of $NaHCO_3$, dialysed against PBS containing the metal ions and lyophilised.

Normal typed human erythrocytes were obtained from S.S.K.M. Hospital, Calcutta. Blood from different species of animals used in this study were procured either from the Insitute Animal House or from other local sources. Erythrocytes were washed in PBS and a 2% cell suspension in PBS was used to assay for activity. Haemagglutination was determined in Plastic microlitre dishes by serially diluting 50 µl samples into 50 µl PBS. Blood cells (50 µl) were added to each well and haemagglutination was usually determined after 60 min. Control wells containing only PBS and red cells were always included. Inhibition of haemagglutination was examined by using several sugars at mM concentrations in PBS. Proteins were determined by Lowry's method (Lowry *et al.*, 1951) using bovine serum albumin as standard. Polyacrylamide gel electrophoresis was carried out on 7.5% gels according to Davis (1964). Total neutral sugar was determined by anthrone method (Spiro, 1966). Leucoagglutination was carried out on a microscope slide using 50 µl leucocytes suspension isolated from 2 ml whole blood and suspended in 2 ml PBS by the method of Boyum (1968). The mitogenic activities of the lectin preparation were measured by assessing their effect on the incorporation of $[^3H]$-thymidine by the cultured human lymphocytes.

Results

Table 1 presents the results of haemagglutination test conducted on the erythrocytes of several species of animals using the crude extract of the seed. No agglutination was observed with erythrocytes of rabbit, guinea-pig, rat, mouse, goat, sheep, cow, buffalow, horse, chicken, pigeon and frog even at a concentration of 500 µg protein. Interestingly, the extract selectively agglutinated human erythrocytes belonging to O, B, A and AB groups. Further, the extract showed highest

Table 1. Haemagglutination test by crude extract of seeds of *Erythrina variegata* Linn. var. *orientalis* Linn. Merrill.

Agglutination	Erythrocytes	Minimum amount of protein required for agglutination (μg)
Positive	Human O	65
	B	140
	A, AB	285

A 2% suspension of washed RBC in PBS was used. Incubation time was 60 min at 37°C. No agglutination was observed with the 2% washed erythrocytes of rat, guinea-pig, mouse, goat, rabbit, dog, cow, sheep, horse, buffalow, chicken, pigeon and frog using 500 μg of protein.

affinity for human 'O' group erythrocytes, at a much lower protein concentration. The purification procedure (table 2) indicates the isolation of the lectin with higher specific activity. The activity as measured by haemagglutination indicated the recovery of 81% of the original activity with a 19-fold purification. Leucoagglutination phenomenon was observed with 3μg purified lectin in 50μl cell suspension within 2–5 min at 37° C suggesting the presence of a strong leucoagglutination property in the lectin along with the haemagglutinating activity. Polyacrylamide disc gel electrophoresis (figure 1) revealed a sharp single protein band. It can be seen that the purified lectin band corresponded to the fastest moving band amongst other proteins of 30–70% $(NH_4)_2SO_4$ saturation fraction under analogous experimental condition. The purified lectin was a glycoprotein containing

Table 2. Summary of purification of the lectin from the seeds of *Erythrina variegata* Linn. var. *orientalis* Linn. Merrill.

Fractions	Total Protein (mg)	Total haemagglutination activity with 'O' group erythrocyte (titre)	Specific* activity	Yield (%)	Purification fold
Crude Extract	2200	7240	3.3	100	1
$(NH_4)_2SO_4$ saturation					
0–30%	800	nil	—	—	—
30–70%	394	6516	16.5	90	5
70–90%	620	200	0.3	—	—
Supernatant	215	nil	—	—	—
Affinity purified fraction	92	5888	64	81	19

* Specific activity was expressed as titre per mg of protein and the titre was defined as the highest dilution showing visible agglutination.

Minimum concentrations of the purified lectin for erythro-agglutination of O, B and A group erythrocytes were in the range of 3–4 μg, 5–7 μg and 10–14 μg respectively.

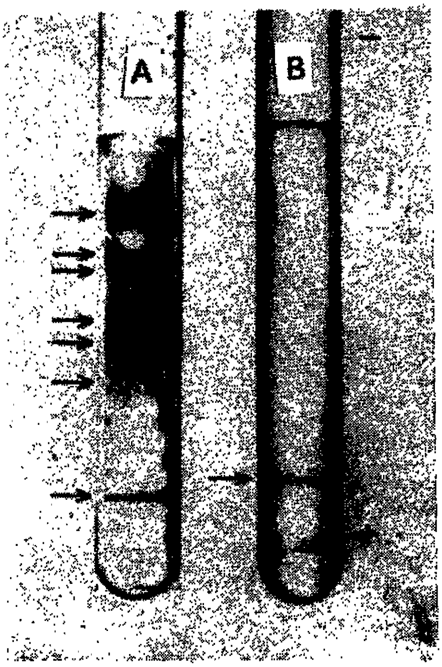

Figure 1. Polyacrylamide gel electrophoresis at pH 8.3 on 7.5% gels, current 5 mA/gel, time 45 min, amount of protein applied 50 μg in each case. Stain: Coomassie brilliant blue in methanol acetic acid mixture. **A.** 30–70% $(NH_4)_2SO_4$ precipitated protein. **B.** Purified lectin.

approximately 10% neutral sugars. A number of mono- and disaccharides were tested for their ability to inhibit the agglutination reaction between the erythrocytes and the lectin (table 3). The haemagglutinating activity of this lectin was completely inhibited by N-acetyl-D-galactosamine, D-galactose and lactose respectively, and the rest of the sugars tested did not elicit any inhibition of the reaction under identical experimental conditions.

Table 3. Effect of various sugars on erythro-agglutination.

Inhibition of agglutination	Sugar	Concentration (mM)
Positive	D-Galactose	3
	Lactose	1.3
	N-Acetyl-D-galactosamine	2

Human O group erythrocytes were used for the haemagglutination assay. 30 min preincubation of 25 μg purified lectin with the test sugar was carried out in each case. Rest of the procedure is same as described in the text.

No inhibitory effect even at 120 mM concentration of D-glucose, D-mannose, fructose, L-fucose, D-arabinose, L-xylose, L-rhamnose, glucopyranoside, methyl-α-D-mannopyranoside, D-glucosamine, D-mannosamine, L-sorbose, sorbitol, D-mannitol, D-glucuromic acid, maltose, sucrose, cellobiose.

Discussion

Some seeds of the genus *Erythrina*, like several others belonging to leguminosae, contained erythroagglutinating activity. Indian coral tree has been attributed with diverse medicinal values in Ayurveda and ancient Hindu medicine (Chopra *et al.*, 1956). In a chemical and pharmacological evaluation of the genus *Erythrina*, the alkaloids of *Erythrina variegata* L. were studied by Ghosal *et al.* (1972). Recently a similar study on *Erythrina mulungu*, which is a medicinal plant used in Folk medicine, has been reported (Sarragiotto *et al.*, 1981). Lee *et al.* (1977) in a survey of lectin in south-east Asian Leguminosae recorded for the first time that *Erythrina variegata* L. agglutinated specifically human erythrocytes of A, B, O and AB blood groups. However, no particular attention was given to this property of the seed extracts. A D-galactose binding lectin, agglutinating human erythrocytes of blood groups O, B, A and AB, was purified from the seeds of Indian coral tree, *Erythrina variegata* L. var. *orientalis* Linn. Merril. by affinity chromatography on acid-treated sepharose 4B gel. The extract exhibited a high affinity for O group erythrocytes and in suitable dilutions specifically identified this blood group. The lectin preferentially agglutinated human erythrocytes, and exhibited a preference in the sequence of O>B>A=AB. The lectin is a glycoprotein having leucoagglutinating property (Datta and Basu, 1981). The purified lectin showed mitogenic activity with human peripheral lymphocytes in preliminary observations. Gilboa-Garber and Mizrahi (1981) isolated a new mitogenic D-galactosephilic lectin from the seeds of coral tree, *Erythrina corallodendron.* The lectin was shown to be a glycoprotein of molecular weight around 110,000–120,000, and exhibited a considerable mitogenic activity towards human lymphocytes (predominantly *T* cells) only after their treatment with neuraminidase. However, in regard to the intensity of their agglutinating activity towards erythrocytes, obtained from different animals, and human donors of diverse ABO blood groups, the lectin strongly agglutinated only untreated human erythrocytes and exhibited a preference for O and B compared with A red blood cells. In a study of D-galactose binding lectins isolated from the seeds *Butea frondosa*, *Erythrina indica* and *Mamordica charantia*, Horejsi *et al.* (1980) showed that the purified *E. indica* lectin agglutinated human group O erythrocytes slightly better than those from B and A groups respectively. The purified lectin was shown to be a metallo-glycoprotein having a molecular weight, 66,200 ± 3000. Bhattacharyya *et al.* (1981) purified non-specific D-galactose binding lectins from some *Erythrina* species. A D-galactose/N-acetyl-D-galactosamine—specific lectin was isolated from *Erythrina crista-galli* and it agglutinated human erythrocytes of all blood types as well as rabbit erythrocytes (Iglesias *et al.*, 1982), and the immunochemical studies on the combining site of the lectin were done by Kaladas *et al.* (1982).

Therefore, the finding of this new lectin with erythro-agglutinating, leucoagglutinating and mitogenic property would be of importance in the field of immunohaematology and both structural and cellular immunology.

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Fluorescence polarization as a tool to study lectin-sugar interaction

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Abstract. The binding of *Ricinus communis* agglutinin and *Abrus* agglutinin to 4-methylumbelliferyl β-D-galactopyranoside was studied by equilibrium dialysis, fluorescence quenching and fluorescence polarization. The number of binding sites and the association constant value obtained by fluorescence polarization for both *Ricinus communis* agglutinin and *Abrus* agglutinin are in close agreement with those obtained by the other methods. This indicates the potential of ligand-fluorescence polarization measurements in the investigation of lectin-sugar interactions.

Keywords. Fluorescence polarization; lectin; sugar binding.

Introduction

Lectins, the carbohydrate binding proteins have proved to be useful tools for probing structural features of cell-surface glycoconjugates (Lis and Sharon, 1973; Rapin and Burger, 1974; Goldstein and Hayes, 1978). The binding of lectins to the carbohydrate moiety of cell membrane receptors is a prelude to a wide range of biological effects of lectins on cells (Cuatrecasas and Tell, 1973; Sharon, 1976). Fluorescently labelled sugars have been used for investigation of the binding parameters of certain lectins such as concanavalin A and Wheat Germ Agglutinin (Dean and Homer, 1973; Privat *et al.*, 1974; Loontiens *et al.*, 1977). On the other hand, lectins such as that from *Pisum sativum* (pea) and *Ricinus communis* (castor-bean), which do not show appreciable ligand fluorescence quenching or enhancement could not be investigated by utilizing these sugars.

During our investigation of 4-methylumbelliferyl β-D-galactopyranoside (MeUmb-Gal$_p$) binding to *R. communis* agglutinin (RCA$_1$) and *Abrus* agglutinin by fluorescence spectroscopy, no appreciable quenching or enhancement of ligand fluorescence was observed in the case of RCA$_1$, whereas it was 30% quenched by *Abrus* agglutinin (Khan *et al.*, 1980, 1981).

Fluorescence polarization of a molecule depends on the molecular size, geometry and rigidity (Weber 1952; Steiner, 1954; Haber and Bennett, 1962; Kierszenbaum *et al.*, 1969). It was anticipated that the interaction of MeUmb-Gal$_p$ with

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Abbreviations used: RCA$_1$, *Ricinus communis* agglutinin; MeUmB-Gal$_p$, 4-methylumbelliferyl β-D-galactopyranoside; MeUmb-Glu$_p$, 4-methylumbelliferyl β-D-glucopyranoside.

RCA$_1$ and *Abrus* agglutinin would lead to alterations in polarization of its fluorescence. Therefore, changes in fluorescence polarization of the sugar on its binding to RCA$_1$ and *Abrus* agglutinin appeared to be an ideal approach to characterize this interaction. A marked change in the polarization of MeUmb-Gal$_p$ fluorescence was observed on binding with RCA$_1$ and *Abrus* agglutinin. The number of binding sites obtained by fluorescence polarization was two for both the lectins. The association constant value for RCA$_1$ ($K_a = 2.9 \times 10^4$ M^{-1}) and *Abrus* agglutinin ($K_a = 1.42 \times 10^4$ M^{-1}) obtained by polarization compares well with those obtained by equilibrium dialysis and other methods.

Materials and methods

4-Methylumbelliferyl β-D-galactopyranoside and 4-methylumbelliferyl β-D-glucopyranoside (MeUmb-Glu$_p$) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. The concentration of the fluorescent sugar solutions was determined at 318 nm using $\varepsilon = 1.36 \times 10^4$ M^{-1} cm^{-1} (Loontiens *et al*, 1977). Lactose was obtained from British Drug House, Poole, Dorset, UK, and all other chemicals used were of analytical grade.

Lectins

Ricinus communis agglutinin (RCA$_1$) and *Abrus* agglutinin were purified by affinity chromatography on cross-linked guar gum (Appukuttan *et al*, 1978) and arabinogalactan (Mazumdar and Surolia, 1978, 1979) respectively. Concentration of RCA$_1$ and *Abrus* agglutinin solutions was determined using

$A_{280}^{1\%, 1\ \mathrm{cm}} = 14$ and $A_{280}^{1\%, 1\ \mathrm{cm}} = 14.6$ (Olsnes *et al*, 1974) respectively.

Equilibrium dialysis

A fixed concentration of RCA$_1$ (50 μM) or *Abrus* agglutinin (23 μM) was placed in one compartment of the dialysis cell and varying concentrations of MeUmb-Gal$_p$ (5–150 μM) in the other compartment. After equilibration at 25° C for 24 h, the concentration of the sugar was determined spectrophotometrically in the protein free compartment and the data analysed according to Scatchard (1949).

Fluorescence titration

A fixed amount (1 ml) of MeUmb-Gal$_p$ (3 μM) was titrated with a 192 μM solution of RCA$_1$ (Khan *et al*, 1980) or 229 μM solution of *Abrus* agglutinin (Khan *et al*, 1981) at 25° C. The fluorescence spectra were recorded in a Perkin-Elmer MPF-44A ratio-recording spectrofluorimeter. Excitations were done at 318 nm and the emission spectra recorded above 330 nm, with a slit of 5 nm for both the monochromaters.

Fluorescence-polarization measurement

Fluorescence-polarization measurements were made on the above spectrofluorimeter equipped with polarizing accessory. Excitation and emission wavelengths were 318 nm and 375 nm respectively. The changes in the fluorescence polarization of a 2.2 μM MeUmb-Gal$_p$ solution on titration against increasing amounts of 192 μM RCA$_1$ solution (Khan *et al*, 1980) and a 2 μM MeUmb-Gal$_p$ solution titrated against increasing amounts of 229 μM *Abrus* agglutinin solution (Khan *et al*,

1981) was recorded. In another set of experiments the concentration of RCA_1 (20 μM) and *Abrus* agglutinin (53.8 μM) was fixed and the polarization of various concentrations of MeUmb-Gal_p were recorded respectively.

Results

The equilibrium dialysis data, when plotted according to Scatchard (1949) as in figure 1 A and B were consistent with two binding sites per molecule of M_r 120,000 and 130,000 for RCA_1 and *Abrus* agglutinin respectively. The association constant obtaind by equilibrium dialysis for RCA_1 and *Abrus* agglutinin were $K_a = 2.6 \times 10^4\ M^{-1}$ and $K_a = 1.62 \times 10^4\ M^{-1}$ at 25° C, respectively.

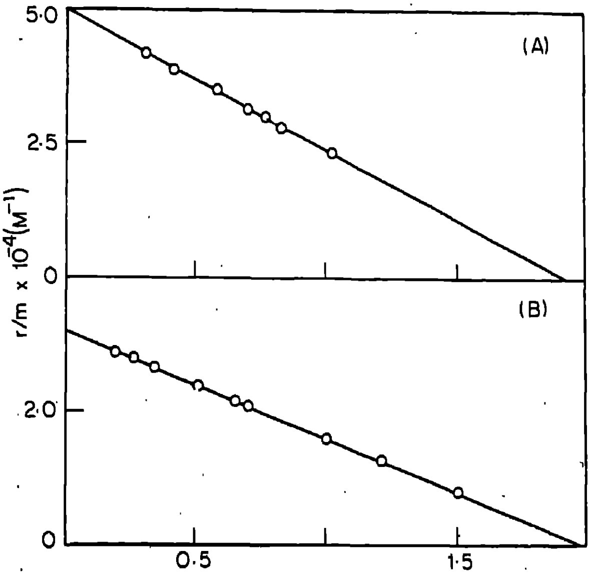

Figure 1. A. Scatchard plot for the binding of MeUmb-Gal_p to *Abrus* agglutinin. **B.** Scatchard plot for the binding of MeUmb-Gal_p to RCA_1. Determined by equilibirum dialysis at pH 7.2, 25° C.

The binding was monitored at 318 nm for MeUmb-Gal_p in the protein-free compartment; γ is the ratio of bound ligand to total protein concentration and *m* is the free MeUmb-Gal_p concentration at equilibrium. From the slope K_a value was calculated. Experiments were carried out in 0.02 M phosphate buffer (pH 7.2) containing 0.1 M NaCl.

The fluorescence of MeUmb-Gal_p even in the presence of large excess of RCA_1 shows an enhancement of only 2.5%, indicating that there is virtually no change in the polar environment of the sugar when it is bound to the lectin. The fluorescence of MeUmb-Gal_p when bound to *Abrus* agglutinin was 30% quenched and was inhibited by lactose, whereas MeUmb-Glu_p fluorescence was not quenched at all. From the quenching titration the association constant of the sugar for the lectin was estimated by the relationship given earlier (Loontiens *et al*, 1977; Khan *et al*, 1981).

$$\log\left(\frac{F_0-F}{F-F_\alpha}\right) = \log K_a + \log\left(|P|t - |M|t\,\frac{(F_0-F)}{F_0}\right)$$

A plot of log $(F_0 - F)/(F - F_\alpha)$ against log p, where p is the free protein concentration given by the log term within the parentheses; assuming 1 : 1 stoichiometry for the binding, with an equivalent weight of 65,000 for *Abrus* agglutinin gave an association constant 1.51×10^4 M^{-1} at 25°C (figure 2A, B).

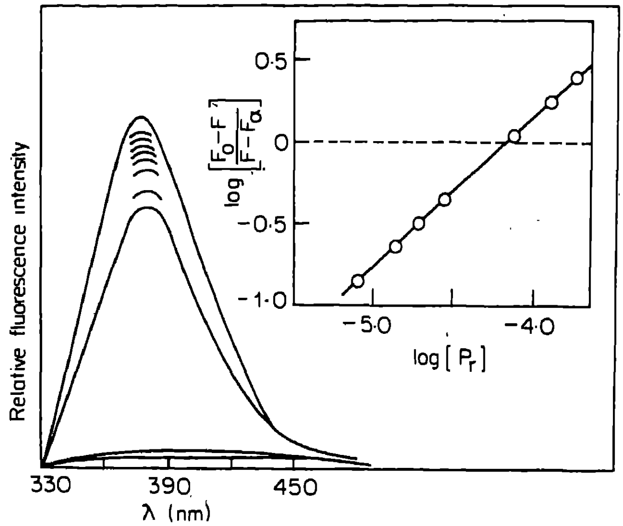

Figure 2. Quenching of the fluorescence of MeUmb-Gal$_p$ by *Abrus* agglutinin.

Experiments were carried out in 0.02 M phosphate buffer (pH 7.2) containing 0.1 M NaCl at 25°C. The fluorescence spectra were recorded in the absence (top curve) and after addition of several aliquots of 229 μM *Abrus* agglutinin to 1 ml of 3 μM solution of MeUmb-Gal$_p$ (30 μl, 60 μl, 90 μl, 120 μl, 320 μl, 530 μl respectively). After each addition of the agglutinin, part of the spectrum was recorded, and the full spectrum is given for the final addition. After correction for dilution, the value of the association constant was determined graphically (inset).

Fluorescence polarization

Polarization, p, of a molecule is expressed quantitatively in terms of intensities (I), polarized either parallel ($\parallel$) or perpendicular ($\perp$) to the incident electric field:

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \tag{1}$$

For a randomly oriented molecule in a rigid medium, the maximum value of p observed with linearly polarized light is 0.5; if the molecules are subject to rotary Brownian motion, the molecular rotation taking place between the time of absorption and emission may be expected to result in values of p between 0.5 and zero. The extent of this rotation is a function of molecular dimensions and structure, solvent and temperature. Low-molecular-weight compounds will give rise to a depolarized fluorescence and thus, for MeUmb-Gal$_p$, the p value is 0.07 at 25° C.

If p, p_B and p_F represent the polarization of the sample, bound sugar and free sugar respectively, the molar fraction of bound sugar (f_B) is given by:

$$f_B = \frac{p-p_F}{p_B-p_F} \quad , \tag{2}$$

from which the molar fraction of free sugar (f_F) can be calculated as:

$$f_F = 1 - f_B = \frac{p_B-p}{p_B-p_F} \tag{3}$$

This will yield

$$\frac{f_B}{f_F} = \frac{p-p_F}{p_B-p} \, , \tag{4}$$

provided the quantum yields of bound and free sugar are the same. If not,

$$\frac{f_B}{f_F} = \frac{Q_F}{Q_B} \; \frac{p-p_F}{p_B-p} \, ; \tag{5}$$

where Q_F and Q_B are the quantum yields of free and bound sugar respectively. The Scatchard equation can be written as:

$$\frac{f_B\,[P]}{f_F} = \frac{n}{K_D} - \frac{f_B[M]}{K_D[P]} \, , \tag{6}$$

where $[M]$ and $[P]$ are the concentration of the sugar and protein respectively; n is the number of sugar molecules bound per molecule of protein and K_D is the dissociation constant. Thus a plot of $f_B P/f_F$ against $f_B\,[M]/[P]$ yields a straight line with intercepts of n/K_D and n on the y and x axes respectively. Of the parameters employed p_F and Q_F are the values for free sugar, whereas p_B and Q_B have to be determined. The values of polarization of fluorescence intensity, when the probe is completely bound to the lectin, will give p_B and Q_B. In case where complete binding cannot be achieved experimentally, p_B and Q_B are obtained by an extrapolation as described for antigen-antibody interaction (Dandliker *et al.*, 1978). If the equilibrium values of p, plotted against concentration $[M]_t$ are extrapolated to zero concentration, where $[M]_t$ is that total molar concentration of the ligand in both free and bound forms; p approaches a limit, p'. The values of p' for different lectin concentrations plotted against $(p'-p_F)$ divided by lectin concentration in any arbitrary convenient unit, p, gives p_B as the intercept of a straight line:

$$p' = p_B - Q_F \, \frac{(p'-p_F)}{Q_{BP}} \tag{7}$$

A similar relationship can facilitate the determination of Q_B:

$$Q' = Q_B + \frac{Q_F - Q'}{p} \tag{8}$$

The changes in the fluorescence polarization of MeUmb-Gal$_p$ on titration of a fixed concentration of RCA$_1$, or *Abrus* agglutinin by various concentration of MeUmb-Gal$_p$ were subjected to Scatchard analysis according to eqn. 6 and the data plotted are shown in figure 3 A and B. At 25° C the value of K_a is, 2.94×10^4 M^{-1} for RCA$_1$ and for *Abrus* agglutinin the K_a is 1.42×10^4 M^{-1}. The number of binding sites for both the lectins is approximately equal to two.

The changes in fluorescence polarization of a 2.2 μM solution of MeUmb-Gal$_p$ on titration with RCA$_1$ (192 μM) is shown in figure 4 A. An increase in ligand fluorescence polarization with increasing RCA$_1$ concentration is observed, reaching a saturation value at 20 μM-RCA$_1$. A complete reversal of the polarization was observed on addition of lactose to lectin fluorescent sugar solution. The changes in fluorescence polarization of a 2.0 μM solution of MeUmb-Gal$_p$ on titration with *Abrus* agglutinin (229 μM) is shown in figure 4 B. An enhancement in the polarization of the ligand fluorescence was also observed with increasing concentration of *Abrus* agglutinin, and it was reversed on addition of lactose.

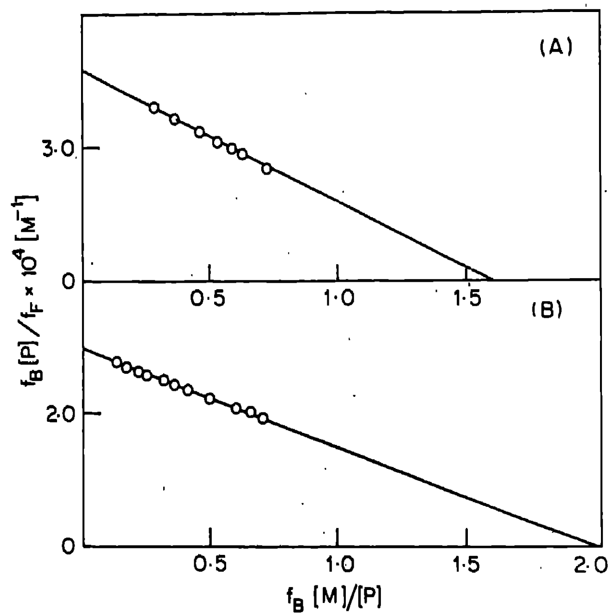

Figure 3. A. Scatchard plot for the binding of MeUmb-Gal$_p$ to RCA$_1$ determined by fluorescence polarization. **B.** Scatchard plot for the binding of MeUmb-Gal$_p$ to *Abrus* agglutinin determined by fluorescence polarization.

RCA$_1$ (20 μM) was titrated with various concentrations of MeUmb-Gal$_p$ (A).

Abrus agglutinin (54 μM) was titrated with various concentrations of MeUmb-Gal$_p$. f_B and f_F are the molar fraction of bound sugar and free sugar respectively. [M] and [P] are the concentration of the sugar and lectin respectively. From the slope, the K_a value was calculated. Experiments were carried out in 0.02 M phosphate buffer (pH 7.2) containing 0.1 M NaCl (B).

From the polarization data of titration of a fixed concentration of MeUmb-Gal$_p$ by various concentration of RCA$_1$ or *Abrus* agglutinin, the association constant can also be obtained by a modified form of the relationship given earlier (Chipman *et al*, 1967).

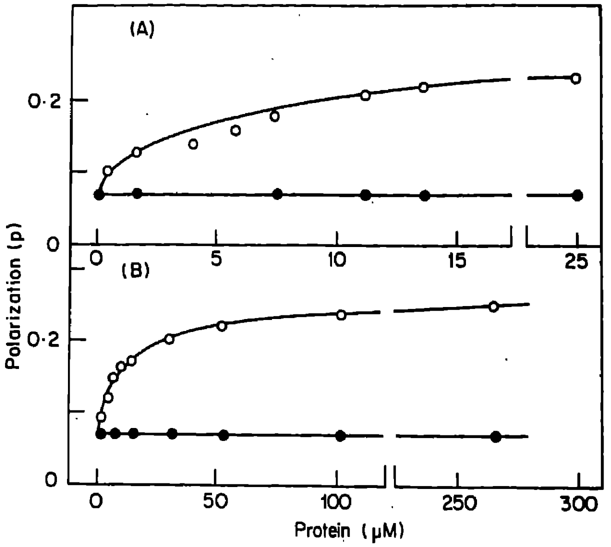

Figure 4. Changes in fluorescence polarization of MeUmb-Gal$_p$ at different concentration of RCA$_1$ and *Abrus* agglutinin.

A. A 2.2 μM solution of MeUmb-Gal$_p$ was titrated with portions of 192 μM RCA$_1$ (O). Titration curve of 2.2 μM solution MeUmb-Gal$_p$ with 192 μM RCA$_1$ solution preincubated with lactose (●).

B. A 2.0 μM solution of MeUmb-Gal$_p$ was titrated with portions of 229 μM *Abrus* agglutinin (O). Titration curve of 2.0 μM MeUmb-Gal$_p$ with 229 μM *Abrus* agglutinin preincubated with 0.1 M lactose (●). Experiments were carried out in 0.02 M phosphate buffer (pH 7.2) containing 0.1 M NaCl at 25°C.

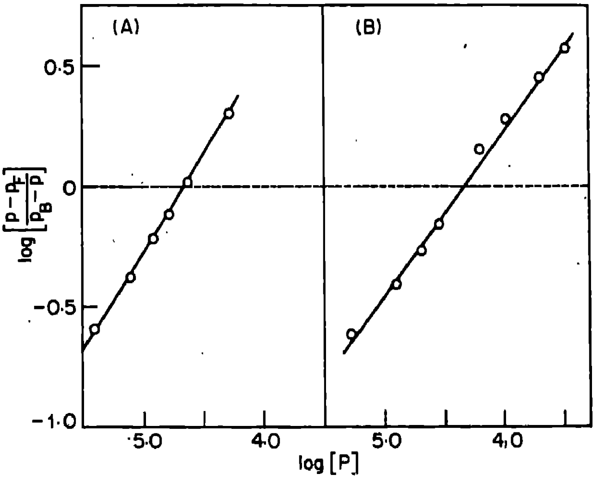

Figure 5. Sample plot of fluorescence polarization data of MeUmb-Gal$_p$ with various concentration of RCA$_1$ (A) and *Abrus* agglutinin (B).

p, p_B and p_F are the relative polarizations of sugar sample, bound sugar and free sugar respectively. $[P]$ is the free protein concentration. From the intercept on the x-axis, K_a was calculated. Experiments were carried out in 0.02 M phosphate buffer (pH 7.2) containing 0.1 M NaCl.

$$\log\left(\frac{p-p_F}{p_B-p}\right) = \log K + \log\left([P]_t-[M]_t\frac{(p-p_F)}{p}\right)$$

The log term within the bracket represents the free protein concentration denoted by log P in figure 5 A and B. A plot of log $(p-p_F)/(p_B-p)$ against log P assuming 1 : 1 stoichiometry for ligand-protein interaction for 60,000 M_r RCA$_1$ or 65,000 M_r *Abrus* agglutinin gave straight line (figure 5 A and B). The slope of the plots was unity, which proved that the assumed stoichiometry was correct. The intercept on the x-axis gave the value of $K_a = 4.2 \times 10^4\ M^{-1}$ for RCA$_1$ and $K_a = 2.14 \times 10^4\ M^{-1}$ for *Abrus* agglutinin.

Discussion

The fluorescence of MeUmb-Gal$_p$ was not quenched nor appreciably enhanced on binding to RCA$_1$, whereas on binding to *Abrus* agglutinin it was quenched by 30%. The reversal of the quenching in case of *Abrus* agglutinin by lactose and no quenching of MeUmb-Glu$_p$ fluorescence indicates that the quenching is due to sugar specific binding only. The total lack of quenching of MeUmb-Gal$_p$ fluorescence by RCA$_1$ indicates a polar environment around the sugar binding site of RCA$_1$ (Delmotte *et al.*, 1975), whereas 30% quenching by *Abrus* agglutinin indicates a less polar environment around its binding site (van Landschoot *et al.*, 1977).

A marked change in the polarization of MeUmb-Gal$_p$ fluorescence was observed on binding to RCA$_1$ or *Abrus* agglutinin. This increase in polarization was utilized for determining the equilibirum parameters for these interactions. The number of binding sites obtained by fluorescence polarization was two for both RCA$_1$ and *Abrus* agglutinin and this is in agreement with the values obtained by equilibrium dialysis and fluorescence quenching. The associaton constant values obtained for RCA$_1$ and *Abrus* agglutinin for MeUmb-Gal$_p$ are consistent with those obtained by equilibrium dialysis, fluorescence quenching and with those reported in literature for other sugars (van Wauve *et al.*, 1973; Podder *et al.*, 1974, 1978; Olsnes, 1978). The Scatchard plots of fluorescence polarisation and equilibrium dialysis are linear in fractional and high saturation ranges, indicating the absence of additional low-affinity sites and lack of interaction between the two identical sites. This is consistent with several studies reported in literature for binding of other lectins to simple carbohydrates and their aromatic glycosides (Hassing and Goldstein, 1970; van Wauve *et al.*, 1973; Podder *et al.*, 1974; van Landschoot *et al.*, 1977). The consistency in the number of binding sites and association constant values obtained for RCA$_1$ and *Abrus* agglutinin by equilibium dialysis, fluorescence quenching and polarization, demonstrates the validity of the fluorescence polarization studies.

The observed alterations in the polarization of ligand fluorescence, that occurs on binding of the fluorescent sugar to the lectins is advantageous, because it provides a direct and rapid measure of the ratio of bound/free sugar. The small amount of material required for these studies is an added advantage. In summary, the present work demonstrates that the fluorescence-polarization measurements can be used for the determination of (a) the number of binding sites, (b) the degree of heterogeneity of the sites and (c) K_a values for the binding of proteins to their ligands. Fluorescence polarization will thus prove a valuable tool in elucidating the mode of interaction of sugars, especially fluorescently labelled sugars, with several other lectins that do not markedly influence the emission of ligand fluorescence.

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Effect of diphtheria toxin fragment A on energy coupling in mitochondria. Studies on mouse liver mitoplasts

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Abstract. The effect of intact diphtheria toxin and of its fragment A on protein synthesis in mouse liver mitoplasts (digitonin-treated mitochondria) was studied. Fragment A inhibited protein synthesis in intact mitoplasts to the same extent as the uncoupler, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, but similar effects were not observed in lyzed mitoplasts. Intact diphtheria toxin was without effect in either case.

Fragment A strongly stimulated mitochondrial ATPase activity. At concentrations which efficiently inhibited mitochondrial protein synthesis and stimulated ATPase activity, fragment A had no effect on the intramitochondrial concentration of nicotinamide-adenine dinucleotides. Moreover, it did not catalyze ADP ribosylation of mitochondrial proteins. The results indicate that the effects observed did not involve the NAD^+-glycohydrolase activity of fragment A.

$[^{125}I]$-Labelled fragment A was bound to mitoplasts to about the same extent as the labelled intact diphtheria toxin.

The present results suggest that fragment A of diphtheria toxin is capable of inhibiting the energy coupling in mitoplasts, thereby inhibiting protein synthesis. The detailed mechanism of the uncoupling and its possible physiological significance remains to be elucidated.

Keywords. Diphtheria toxin; fragment A; mitochondria; mitoplasts; uncoupling.

Introduction

Recently we have been involved in a study of protein synthesis in mouse liver mitochondria in an attempt to characterize the polypeptides formed. For this purpose mitoplasts, *i.e.* mitochondria devoid of its outer membrane, were used. In order to ascertain that the polypeptides formed were genuine mitochondrial products, the effect of various inhibitors, such as chloramphenicol and cycloheximide, was tested (Ashwell and Work 1970; Schatz and Mason, 1974). The sensitivity to diphtheria toxin was used as an additional criterion since this toxin is known to be a potent inhibitor of the cytoplasmic elongation factor 2 (Gill *et al.*, 1969), but not of the corresponding factor from yeast mitochondria (Richter and Lipmann, 1970). In agreement with the above finding, protein synthesis in mammalian mitoplasts was not inhibited by intact diphtheria toxin. Since the amount of fragment A which carries the toxic activity, varies in different toxin preparations, we tested the effect

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Abbreviations used: DTT, Dithiothreitol.

of purified fragment A in this system. Unexpectedly, fragment A, in contrast to intact diphtheria toxin, inhibited protein synthesis in mitoplasts. Here we report these results as well as studied designed to elucidate the nature of the effect. The data presented indicate that the inhibitory effect of fragment A on protein synthesis in mitoplasts is indirect, and due to a previously unkown property of fragment A, *viz.* the ability to inhibit energy coupling.

Materials and methods

Materials

Diphtheria toxin was obtained from Connaught Laboratories Ltd., Ontario, Canada, digitonin from Sigma Chemical Co., St. Louis, Missouri, USA and [^{35}S]-methionine and [^{14}C]-NAD$^+$ from The Radiochemical Centre, Amersham, England.

Preparation of mitoplasts

All solutions used for the preparation of mitoplasts were sterile filtered and all centrifugations were carried out at 2° C in a Sorvall HB-4 rotor, using sterile 30 ml Corex tubes. Liver mitochondria were isolated from 6 weeks old Swiss mice, (Romslo and Flatmark, 1973). The final mitochondrial pellet was suspended in Hepes sucrose medium at a final concentration of 7.5 mg protein/ml, and treated with a 3 mg/ml solution of digitonin in the same buffer to a final concentration of 0.2 mg digitonin/mg protein (Chan *et al.*, 1970). The digitonin-treated mitochondria were slowly stirred at 2° C for 15 min and diluted with Hepes sucrose medium to a final concentration of 5 mg protein/ml. The mitoplasts were pelleted by centrifugation at 10,500 *g* for 10 min. The pellet was resuspended in 20 ml of Hepes sucrose medium and centrifuged at 500 g for 5 min. Mitoplasts in the supernatant were pelleted by centrifugation at 7,000 g for 10 min and suspended in a buffer containing 5 mM Hepes (pH 7.4) and 150 mM KCl at a final protein concentration of 60 mg/ml. The mitoplasts were used immediately after preparation.

Isolation of the mitochondrial matrix and inner membrane fraction

Mitoplasts were resuspended in Hepes sucrose and sonicated in a pyrex tube (5 ml) cooled in ice-water. The sonifier (Branson, model S-75) was operated at a current output of 4A, and the suspension was sonicated five times for 30 s with 1 min intervals in order to keep the temperature below 10° C. The sonicated mitoplasts were centrifuged at 165,000 *g* for 2 h, using the 50 Ti rotor (fixed angle, $R_{min} = 3.8$ cm and $R_{max} = 8.1$ cm) of the Spinco L2-65 B ultracentrifuge, operated at 4° C. The matrix fraction (supernatant) and the inner membrane fraction (pellet) were collcted.

Preparation of diphtheria toxin fragment A

Fragment A of the toxin was prepared by trypsin treatment of intact diphtheria toxin, followed by dissociation with dithiothreitol (DTT), according to Drazin *et al.* (1971). The A-fragment was dialyzed against 10 mM Hepes buffer (pH 7.4) contaning 1 mM DTT.

Assay of protein synthesis in mitoplasts

Incubations of mitoplasts for measurement of protein synthesizing activity were carried out in open tubes with free access to oxygen. Incorporation of [^{35}S]-methionine into proteins insoluble in hot trichloroacetic acid, was assayed under maximally energized conditions, *i.e.* respiration was supported by NADH-linked substrates and ATP plus ADP were present in the incubation medium. For experimental details, see legend to figure 1.

Assay of ATPase activity

Mitoplasts were incubated at 37° C under conditions used for protein synthesis, with the following modifications: Amino acids were omitted from the reaction mixture which contained 3 mM MgATP. The reaction was stopped after various time intervals by withdrawing 30 μl aliquots of the mixture into 200 μl 5% (v/v) cold perchloric acid. The protein precipitate was removed by centrifugation. The supernatant was diluted (three times), with 0.45 M potassium phosphate, pH 3.93, and 20 μl aliquots of the supernatants were used for analysis of adenine nucleotides.

The adenine nucleotides were assayed by high-performance liquid chromatography (HPLC) as described (Normann and Flatmark, 1978; Terland *et al.*, 1979). The chromatographic separation of the nucleotides was achieved at 20° C on a strong anion exchanger (Partisil 10-SAX), prepacked from Whatman in a 25 cm stainless steel column of 4.6 mm inner diameter. The details are given in legend to figure 3.

Assay of ADP-ribosylating activity

The ability of fragment A of diphtheria toxin to ADP-ribosylate mitochondrial and cytosolic proteins was assayed by measuring the incorporation of radioactivity from [^{14}C]NAD$^+$, labelled in the adenosine moiety, into trichloroacetic acid-insoluble material, according to the procedure of Gill and Dinius (1973).

Assay of toxin binding

Two mg of diphtheria toxin was iodinated, using lactoperoxidase as described earlier (Sandvig *et al.*, 1976). The iodinated protein was purified by molecular sieve chromatography (twice) in 50 mM Tris-HCl buffer. The first chromatography was carried out in the presence of 1 mM NaI. A portion of the iodinated protein was mixed with non-radioactive toxin and fragment A was isolated as described above. Toxin binding was measured by incubating mitoplasts with radioactive toxin under the conditions used for protein synthesis. After 15 min incubation, mitoplasts were pelleted and washed twice with 1 ml of Hepes sucrose medium. Samples were processed for radioactivity measurement as described above.

Measurement of the concentration of nicotinamide adenine dinucleotide in mitoplasts

Mitoplasts were incubated under the conditions described above for the ATPase assay, except that ATP was not added to the reaction mixture. The perchloric acid extract was neutralized to remove the perchlorate. The total concentration of the oxidized and reduced forms of nicotinamide adenine dinucleotide was estimated spectrophotometrically after reduction with yeast alcohol dehydrogenase (Vallee and Hoch, 1955).

Results

Effect of fragment A on protein synthesis in mitoplasts

Intact mitochondria are contaminated to a variable extent by cytoplasmic ribosomes attached to the outer mitochondrial membrane and by fragments of the rough endoplasmic reticulum. Therefore, in all experiments reported here we have used mitoplasts, *i.e.* mitochondria where the outer membrane was removed by treatment with digitonin (Chan *et al.*, 1970). It has been shown that liver mitoplasts prepared in this way are free of membrane fragments from the endoplasmic reticulum and exhibit tight energy coupling (Schnaitman and Greenawalt, 1968) (see also figure 3).

The incorporation of [^{35}S]-methionine into protein by mitoplasts was almost linear for 30 min and thereafter declined. The time course of the reaction rules out the possibility of bacterial contamination of the system. It is also seen that the incorporation was insensitive to cycloheximide, but sensitive to chloramphenicol, as expected for mitochondrial protein synthesis.

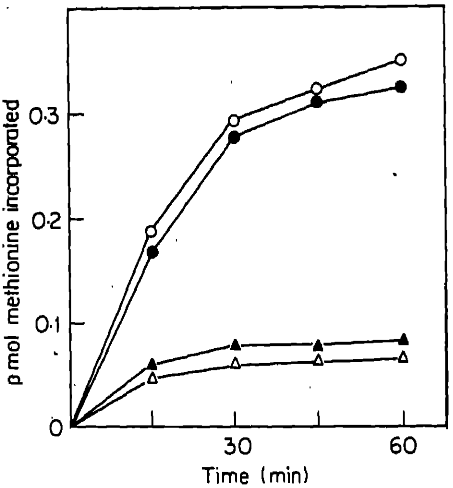

Figure 1. Inhibition of protein synthesis in mitoplasts by diphtheria toxin fragment A. Mitoplasts (300 μg), prepared as described under Materials and methods, were incubated for increasing periods of time at 37°C in a final volume of 50 μl. The incubation mixture contained 40 mM Hepes (pH 7.4), 70 mM KCl, 2 mM Mg^{2+}, 1.2 mM Na_2HPO_4, 5 mM glutamate, 5 mM malate, 1 mM DTT, 1 mM ADP, 0.1 mM ATP, 1 μM each of 19 amino acids and 30 μCi [^{35}S]-methionine (specific activity 900 Ci/mmol). Inhibitors were present in the concentrations given below. At the time points indicated 5 μl samples were withdrawn into 500 μl 0.1 N NaOH, containing 1 mM methionine and 100 mM mercaptoethanol. After 3 min, protein was precipitated with 2 ml of 10% trichloroacetic acid. After heating for 30 min at 90°C, the precipitate was collected on glass fiber filters, washed 10 times with 10 ml of 10% trichloroacetic acid and counted. The radioactivity incorporated is expressed as pmol methionine/mg protein. One pmol methionine corresponds to 1.6×10^6 cpm. (▲), Diphtheria toxin fragment A, 200 μg/ml; (△), chloramphenicol, 200 μg/ml; (●), cycloheximide, 50 μg/ml; (○), control.

The interesting fact emerging from these experiments is that fragment A inhibited protein synthesis in mitoplasts to about the same extent as did chloramphenicol (figure 1). In contrast, in all experiments the complete toxin gave a slight stimulation of the amino acid incorporation. It should be noted that the concentration of fragment A, required to inhibit protein synthesis by 90% was about one order of magnitude higher in mitoplasts than in extracts of HeLa cells (Collier and Pappenheimer, 1964).

In figure 2 the inhibition of protein synthesis in mitoplasts exposed for 15 min to increasing concentrations of fragment A is shown. The dose-response relationship reveals saturation kinetics at high toxin concentration, indicating that in this system fragment A does not inactivate catalytically a component of the protein synthesizing machinery, as is the case with cytoplasmic protein synthesis.

Inability of fragment A to inhibit protein synthesis in lyzed mitoplasts

If the inhibition of mitochondrial protein synthesis by fragment A were the result of a catalytic inactivation of elongation factor 2, as is the case in the cytoplasm (Gill *et al*, 1969), fragment A should inhibit protein synthesis also in lyzed mitoplasts. The protein synthesizing activity of lyzed mitoplasts was found to be rather low, compared to that of intact mitoplasts. However, in the presence of an efficient ATP regenerating system, the lyzed mitoplasts synthesized proteins at about 1/3 the

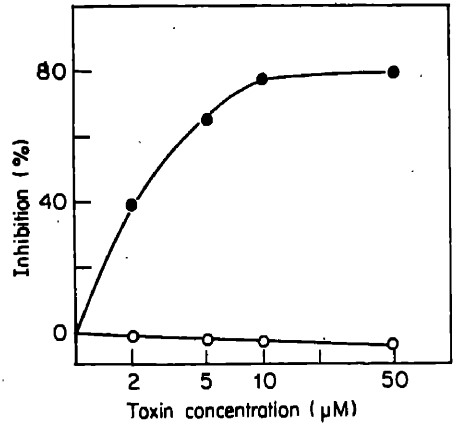

Figure 2. Inhibition of protein synthesis in mitoplasts pretreated with diphtheria toxin. Mitoplasts (1.6 mg), prepared as described under Materials and methods, were incubated with increasing concentrations of complete diphtheria toxin or its fragment A at 37°C for 15 min in a final volume of 200 μl. The incubation mixture contained 40 mM Hepes (pH 7.4), 70 mM KCl, 2 mM Mg^{2+}, 1.2 mM Na_2HPO_4, 5 mM glutamate, 5 mM malate, 1 mM ADP, 0.1 mM ATP and 1 mM DTT. At the end of the incubation period the reaction mixture was diluted with 1 ml of cold Hepes sucrose medium and the mitoplasts were sedimented at 2° C. The pellet was resuspended in 200 μl of fresh incubation mixture containing all the components described above, except toxin. Fifty μl of this mixture was incubated with 1 μM each of 19 amino acids and 30 μCi of [^{35}S]-methionine (specific activity 900 Ci/mmol) at 37°C for 40 min. Five μl samples were processed in duplicate for radioactivity measurement, as described in legend to figure 1. Control mitoplasts were treated in the same way, except that toxin was omitted. The data are expressed as per cent inhibition of protein synthesis. (●), Fragment A of diphtheria toxin; (○), complete toxin.

Table 1. Effect of diphtheria toxin on protein synthesis in lyzed mitoplasts.

Conditions	[^{35}S]-Methionine incorporated/mg protein
	cpm × 10^{-4}
Lyzed mitoplasts	
No addition	22.1
Intact diphtheria toxin	22.4
Fragment A of diphtheria toxin	22.0
Cycloheximide	20.5
Chloramphenicol	4.2
Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone	18.9
Intact mitoplasts	68.2
Fragment A of diphtheria toxin	14.3
Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone	16.8

Mitoplasts suspended in a buffer containing 5 mM Hepes (pH 7.4) and 150 mM KCl were lyzed by adding a 2% (w/v) solution of Brij 58 to a final concentration of 0.1%. After 5 min at 2° C the clear lyzed mitoplasts, equivalent to 300 µg protein, were incubated at 37°C in a buffer solution (final volume 50 µl) with the following additions: 40 mM Hepes (pH 7.4), 70 mM KCl, 2 mM Mg^{2+}, 1 mM ATP, 10 mM creatine phosphate, 2 units of creatine phosphokinase, 1 µM each of 19 amino acids and 30 µCi [^{35}S]-methionine (specific activity 900 Ci/mmol). The concentration of diphtheria toxin and fragment A, when present, was 200 µg/ml. The concentrations of cycloheximide, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone and chloramphenicol were 50 µg/ml, 1.6 µg/ml and 200 µg/ml, respectively. After 40 min incubation, 5 µl samples in duplicate were processed for radioactivity measurements. The data represent the average of three experiments.

rate observed in intact mitoplasts (table 1). Also, under these conditions protein synthesis was insensitive to cycloheximide, whereas chloramphenicol inhibited the process by 80%, demonstrating that the activity measured indeed represents true mitochondrial protein synthesis. It can be seen that fragment A of diphtheria toxin did not inhibit protein synthesis in the lyzed system indicating that the inhibition observed in intact mitoplasts is caused by an indirect mechanism. Interestingly, the uncoupling agent, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone inhibited the protein synthesis in intact mitoplasts to about the same extent as did fragment A (table 1). This finding clearly suggested that the observed effect of fragment A might be due to inhibition of the energy coupling process.

Stimulation of mitochondrial ATPase activity by fragment A

It is well established that uncoupling of oxidative phosphorylation and other energy-dependent processes stimulate the mitochondrial ATPase activity. The effect of fragment A on the ATPase activity of mitoplasts was therefore examined. It is apparent from figure 3 that fragment A very efficiently stimulated the mitochondrial ATPase activity. In fact, fragment A was almost as effective as carbonylcyanide *p*-trifluoromethoxyphenylhydrazone in this regard (data not shown). On the other hand, intact toxin reduced slightly the ATPase activity. The results provide evidence that fragment A inhibits protein synthesis in mitoplasts by inhibiting the energy coupling process.

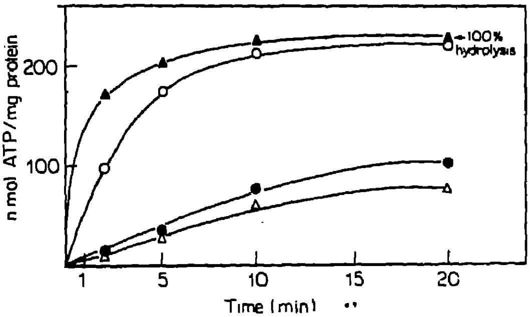

Figure 3. Stimulation of mitochondrial ATPase activity by fragment A of diphtheria toxin. Mitoplasts (2 mg), were incubated at 37°C for 10 min in a final volume of 200 µl. The incubation mixture contained: 40 mM Hepes (pH 7.4), 70 mM KCl, 3 mM Mg^{2+}, 1.2 mM Na_2HPO_4, 5 mM glutamate, 5 mM malate and 1 mM DTT. The concentrations of complete toxin and fragment A were 2.5 mg/ml and 200 µg/ml, respectively. At the end of the incubation the reaction mixtures were diluted with 1 ml of Hepes sucrose medium and the mitoplasts were sedimented at 2°C. The pellet was resuspended in 200 µl of fresh incubation mixture containing all the components described above, except mitoplasts and toxin, and incubated with 3 mM ATP at 37°C. At the time points indicated 30 µl samples were withdrawn into 200 µl of 5% cold perchloric acid. The protein precipitate was removed by centrifugation. The supernatant was diluted three fold with 0.45 M potassium posphate, pH 3.93. Adenine nucleotides in the 20 µl aliquots of the supernatants were analyzed by high performance liquid chromatography. The chromatographic separation of the nucleotides was achieved at 20°C on a strong anion exchanger (Partisil 10-SAX), prepacked from Whatman in a 25 cm stailness steel column of 4.6 mm inner diameter. The mobile phase, consisting of 0.45 M potassium phosphate, pH 3.93, was pumped at a flow rate of 2 ml/min (1,800 p.s.i.), giving the following retention times: t_R(AMP) – 2.02 min, t_R(ADP) – 3.14 min and t_R(ATP) – 11.70 min. The ATPase activity is expressed as nmol ATP hydrolyzed per mg protein. (●), Control; (Δ), complete toxin; (○), fragment A; (▲), control mitoplasts lyzed with 0.2% triton.

Binding of fragment A to the mitochondrial inner membrane

In attempts to elucidate the mechanism by which fragment A inhibits protein synthesis and stimulates ATPase activity in mitoplasts, experiments were performed to determine its binding to and possible penetration of the mitochondrial inner membrane.

From table 2 it is seen that [^{125}I]-labelled fragment A, as well as labelled intact toxin, bind to intact mitoplasts. The binding capacity appeared to be very similar for the two polypeptides, indicating that the stimulatory effect of fragment A on the ATPase activity cannot be explained by selective binding to the inner membrane. In both cases most of the bound radioactivity was found in the membrane fraction. Since the radioactivity present in the matrix fraction was greater after incubation with the complete toxin, which was inactive in the system, than after incubation with fragment A, entry of fragment A into the matrix is apparently not required for the inhibition of the mitochondrial protein synthesis.

Role of enzymatic activity of fragment A

Since fragment A has NAD^+-glycohydrolase activity (Collier, 1975), its effect on the intramitochondrial concentration of NAD^+ and NADH was measured. It is

Table 2. Binding of radioactive diphtheria toxin and fragment A to mitoplasts.

Additions	Radioactivity bound/mg protein		
	Intact mitoplast	Membrane fraction	Matrix fraction
	cpm	cpm	cpm
Complete toxin	5,860	10,200	1,560
Fragment A	4,950	10,800	502

[^{125}I]-Labelled diphtheria toxin (1×10^5 cpm), and fragment A (1×10^5 cpm) were incubated for 15 min at 37°C with 3 mg mitoplasts in a final volume of 200 µl under the conditions used for protein synthesis (legend to figure 1), except that labelled methionine was replaced by non-radioactive methionine. Mitoplasts were pelleted at 2°C and washed twice with 1 ml of Hepes sucrose medium. Membrane proteins and matrix proteins were separated by centrifugation of sonicated mitoplasts at 100,000*g* for 1 h. The pellet of intact mitoplasts and membrane fraction was dissolved in 200 µl of 0.1 N NaOH. Twenty µl of dissolved protein and supernatant fraction was precipitated in duplicate with 10% trichloroacetic acid. The precipitate was collected on glass fiber filters for radioactivity measurement.

Table 3. Estimation of the concentration of pyridine nucleotides in mitoplasts exposed to fragment A.

Treatment	Total pyridine nucleotides
	nmol·mg^{-1} protein
None	12.1
Fragment A	11.8

Mitoplasts (4.2 mg) were incubated at 37°C for 15 min in a final volume of 400 µl. The incubation mixture contained: 40 mM Hepes (pH 7.4), 50 mM KCl, 2 mM Mg^{2+}, 1.2 mM Na_2HPO_4, 5 mM glutamate, 5 mM malate and 1 mM DTT. The concentration of fragment A, when present, was 200 µg/ml. The reaction was stopped by the addition of 2 ml of cold 5% perchloric acid. After 15 min at 2°C, the mixture was neutralized with cold 1 M potassium bicarbonate and the precipitate was removed by centrifugation. The supernatant was concentrated, and the total pyridine nucleotide concentration was estimated after reduction with alcohol dehydrogenase in presence of ethanol. The concentrated nucleotide fraction was incubated for 30 min at 25°C in a final volume of 1 ml. The incubation mixture contained 20 mM glycine buffer (pH 8.7), 75 mM sodium phosphate, 10 µl ethanol, 75 mM semicarbazide (pH 8.7) and 10 µg yeast alcohol dehydrogenase (specific activity 150 units/mg). At the end of the incubation, the absorption at 340 nm was measured and the concentration of NADH was calculated, using the molar extinction coefficient $\varepsilon = 6.22 \times 10^3$. The results represent the average of two experiments.

seen from table 3 that the level of NAD$^+$ in the mitoplasts did not change when they were incubated with fragment A at concentrations that efficiently inhibited protein synthesis and stimulated ATPase activity. The data are consistent with the above finding (table 2) that most of fragment A binds to mitoplasts and can be recovered in the isolated inner membrane fraction.

To study the possibility that fragment A might exert its effect on mitochondrial protein synthesis by catalyzing ADP-ribosylation of mitochondrial proteins, we incubated intact mitoplasts and their subfractions with [^{14}C]-labelled NAD$^+$, using liver cytosol as control. From table 4 it is seen that no ADP-ribosylation of mitochondrial proteins was detected.

Table 4. Ability of diphtheria toxin fragment A to ADP-ribosylate proteins from different subcellular fractions.

Additions	Diphtheria toxin fragment A	Radioactivity incorporated
		cpm
Cytosol	+	1145
	−	39
Nuclear fraction	+	204
	−	212
Intact mitoplast	+	38
	−	26
Membrane fraction	+	23
Matrix fraction	+	28
Membrane + matrix	+	32

Mitoplasts (7 mg) were lyzed by sonication and the suspension was centrifuged for 1 h at 10,000 *g* to prepare the membrane (pellet) and the matrix (supernatant) fractions. The membrane fraction was resuspended in 200 μl buffer (50 mM Tris-HCl, pH 8, 70 mM DTT and 0.1 mg/ml bovine serum albumin). The supernatant as well as the membrane fraction were dialyzed against 500 ml of the buffer for 3 h. The nuclear fraction from mouse liver was lyzed with 0.2% sodium deoxycholate. Lyzed nuclear fraction and cytosol fraction from mouse liver were dialyzed against the buffer before use. Each fraction, contaning 200 μg of protein, was incubated with 20 μl of [^{14}C]-NAD$^+$, labelled in the adenosine moiety, and 10 μg of diphtheria toxin fragment A at 37°C in a final volume of 100 μl. Twenty μl samples were withdrawn in duplicates at 0 min and 10 min, precipitated and washed with 5% trichloroacetic acid for radioactivity measurements. The 0-min values were subtracted from each sample. In the sample containing both mitoplast membrane and matrix protein, the total protein concentration in the reaction mixture was 200 μg. The results represent the average of 5 experiments.

Discussion

The present finding that fragment A of diphtheria toxin efficiently inhibits protein synthesis in mitoplasts from mouse liver was unexpected since the intact toxin is unable to inhibit protein synthesis in mitoplasts (figure 2) as well as in yeast mitochondria and prokaryotes (Richter and Lipmann, 1970). The mechanism of the inhibition is, however, different from that operating in the cytoplasm where fragment A inhibits elongation factor 2 by ADP ribosylation (Gill *et al.*, 1969). This follows from the findings that fragment A had no inhibitory effect on the protein synthesis in lyzed mitoplasts containing an ATP regenerating system (table 1) and that no NAD$^+$-glycohydrolase activity or ADP ribosylation of mitochondrial proteins could be detected (tables 3 and 4). It is clear that fragment A has no direct effect on the mitochondrial protein synthesis as such and that the effect of protein synthesis in mitoplasts is brought about by an indirect mechanism. The ability of fragment A to inhibit protein synthesis in intact, but not in lyzed mitoplasts may be used as an additional criterion of specificity for mitochondrial protein synthesis.

In the present study the [^{35}S]-methionine incorporation into acid-insoluble material was assayed in the presence of added external adenine nucleotides and under maximally energized conditions, where mitochondrial respiration was supported by NADH-linked substrates. Previously Wheeldon and Lehninger (1966) have observed that uncouplers of oxidative phosphorylation efficiently inhibit respiration-dependent mitochondrial protein synthesis. In agreement with this it was found that carbonylcyanide *p*-trifluoromethoxyphenylhydrazone inhibited protein synthesis in mitoplasts as efficiently as did fragment A. The present results can therefore be explained by an uncoupling effect of diphtheria toxin fragment A. Direct evidence that fragment A indeed uncouples oxidative phosphorylation, was given in figure 3 where it was shown that fragment A stimulates strongly the ATPase activity of mitoplasts. The finding that after sonication, labelled fragment A could be almost completely recovered from the membrane fraction (table 2), is consistent with a tight binding of this peptide to the mitochondrial inner membrane.

In view of the fact that most diphtheria toxin preparations contain some nicked toxin which may dissociate to give fragment A, it may seem surprising that diphtheria toxin had no inhibiting effect on protein synthesis in the mitoplasts. One possible explanation is, that in the present incubation system, it was necessary to keep the concentration of reducing agent (DDT) below 2 mM, to avoid inhibition of protein synthesis. Conceivably, the low concentration of DDT used was insufficient to release fragment A from nicked toxin molecules.

The question arises, whether the uncoupling effect of fragment A may play a role in the intoxication of cells by diphtheria toxin. This seems unlikely in view of the fact that fragment A inhibits cellular protein synthesis by a catalytic mechanism. Thus, the presence in the cytosol of a single molecule of diphtheria toxin is sufficient to kill cells in culture (Pinchot, 1950). In contrast, the uncoupling effect of fragment A is non-catalytic. Therefore, since each cell contains numerous mitochondria, a large number of molecules is required to inhibit significantly oxidative phosphorylation. Nevertheless, it cannot be ruled out that, in intact animals treated with large doses of diphtheria toxin, the effect of fragment A on oxidative phosphorylation may also be expressed before the animal succumbs to the protein synthesis inhibition. In fact, it has been reported that increased oxygen consumption and reduced levels of high energy phosphate compounds may be observed in mitochondria isolated from the liver and kidneys of pigs treated with intact diphtheria toxin (Pinchot, 1950; Niselovskaya and Paderina, 1978).

Irrespective of its possible role in intoxication of cells, the ability of fragment A to uncouple phosphorylation seems to be of considerable interest. It is known that the linear oligopeptide, gramicidin, uncouples oxidative phosphorylation (Hotchkiss, 1944) by forming ion conducting channels when inserted into the mitochondrial inner membrane (Moore and Pressman, 1964). Whether fragment A acts by a similar mechanism is not evident from the present data. It can be said with reasonable certainty that the uncoupling effect of fragment A is unrelated to its well known enzymatic activity. It seems likely that fragment A acts by altering the properties of the mitochondrial inner membrane. Clearly, further work is needed to elucidate its mechanism of action.

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Evidence for the involvement of exposed surface carbohydrates of *Agrobacterium tumefaciens* in relation to crown gall tumour induction

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Abstract. The exposed carbohydrate residues on the cell surface of both tumourigenic and nontumourigenic strains of *Agrobacterium* have been investigated using lectins as probes. N-acetyl-D-galactosamine and β-D-galactose were found to be present as the exposed groups on the cell surfaces of the *Agrobacterium* strains. These carbohydrate residues are attached to lipids on the outer membrane of the bacteria as lipopolysaccharides. Fluorescently labelled lectins were used to observe bacterial agglutination in fluorescence microscope. The involvement of these exposed carbohydrate groups in host-pathogen interaction was demonstrated by actual inhibition of tumour initiation at wound sites on host plant.

Keywords. β-D-Galactose; N-acetyl-D-galactosamine polygalacturonate; adherence; lectin binding; tumour inhibition (*Agrobacterium tumefaciens*).

Introduction

Agrobacterium tumefaciens induces crown gall tumour in a wide variety of dicotyledonous plants. In order to be virulent *Agrobacterium* strains must harbour a tumour inducing plasmid (T_i) (van Larebeke *et al.*, 1974; Watson *et al.*, 1975; Thomashow *et al.*, 1980). This plasmid is introduced into the host plant genome for the induction and maintenance of tumours by the virulent strains (Chilton *et al.*, 1977; Thomashow *et al.*, 1980; Willmitzer *et al.*, 1980; Yadav *et al.*, 1980). Lippincott and Lippincott (1977, 1980) have shown that the primary event in crown gall tumour induction is the attachment of the bacterial cells to the host plant cell wall. This bacterial adherence has been confirmed by various groups of workers using different methods including electron microscopy (Manigault, 1970; Nissen, 1971; Matthysse, 1978; Smith and Hindley, 1978 Ohyama *et al.*, 1979). Whatley *et al.*, (1976) have demonstrated that *Agrobacterium* component involved in the microbial-plant adherence is the polysaccharide or O-antigen portion of *Agrobacterium* lipopolysaccharide (LPS) by its ability to inhibit tumour induction. The lipid-A portion solubilized with bovine serum albumin is noninhibitory. This moiety anchors the LPS molecule in the outer membrane, while only the projecting polysaccharide chains participate in the adherence. On the part of the host cell wall, polygalacturonic acid has been implicated in the adherence mechanism because of its excellent ability to inhibit tumour induction. A similar situation prevails in the case of the other genera of *Rhizobiaceas* *i.e.* of *Rhizobium* species which induce atmospheric fixing nodules in leguminous plants. Here, the bacterial polysaccharides occurring in the capsule or LPS portion of the envelopes which are antigenically very similar to polysaccharides of the host cell surface, are involved in adherence. Multivalent lectins of the host legume are envisaged to bind to each of these common antigens thereby leading to adherence (Hamblin and Kent, 1973Bohlool and Schmidt, 1974; Dazo and Brill, 1978, 1979). However, in the case of *Agrobacterium* plant interaction, no evidence for the participation of host lectins has been found. A direct polysaccharide-polysaccharide interaction occurs in the adherence of the *Agrobacterium* cells to the host plant cell wall. The polysaccharide groups of *Agrobacterium* cells participating in interaction have been reported (Banerjee *et al.*, 1981). In order to confirm the involvement of N-acetyl-D-galactosamine and β-D-galactose in adherence, lectin binding studies and tumour inhibition studies were carried out.

Materials and methods

Lectins

The following plant lectins were used:

Lens culinaris (D-mannose, D-glucose) was prepared by the method of Howard *et al.* (1971); *Ricinus communis* (β-D-galactose) was purchased from Sigma Chemical Co., St. Louis, Missouri, USA; *Dolichos biflorus* (N-acetyl-D-galactosamine) was prepared by the method of Etzler (1972) and *Ulex europaeus* (D-fucose) was prepared by the method of Osawa and Matsumoto (1972). The carbohydrate specificities of the lectins are given earlier (Thomashow *et al.*, 1980; van Larebeke *et al.*, 1974).

Organisms: The strains of *Agrobacterium tumefaciens* used were (i) TIP Kerr 14 agrocin sensitive, referred to as '*N*' hereafter, (ii) TIP Kerr 14 agrocin resistant, probably cured of plasmid, referred to as '*R*' hereafter and (iii) S 1005 agrocin resistant variety, referred to as '*S*' hereafter. The above strains were kindly provided by Prof. J. Schell, Director, Laboratorium voor Genetica, Rijks Universiteit, Ghent, Belgium.

Growth Media: The organisms were grown in Rich yeast tryptone medium having the following composition 0.8% Bactotryptone, 0.5% Bactoyeast extract and 0.5% NaCl, pH 7.4. The growth temperature was 28° C. Cells from mid log phase were centrifuged at 700 *g* for 20 min in a refrigerated centrifuge (model B 20-A, IEC, Massachusetts, USA), washed twice in 0.1 M phosphate buffered saline (PBS) and resuspended in the same for use in agglutination tests.

Agglutination tests: Serial two fold dilutions of lectins in saline were performed in microtiter plates. Bacterial suspension (25 μl) was added to each well. After thorough mixing the plates were incubated at 37° C for 35 min. Agglutinations were observed and scored under light microscope. Agglutination titres of lectins are given as the reciprocal of the highest dilution, causing agglutination visible under a light microscope.

Pronase treatment: Cell suspensions were treated with 200 μg of pronase (Sigma Chemical Co., St. Louis, Missouri, USA) per ml for 1 h at 37° C. This was then centrifuged at 7000 *g* for 30 min, washed twice with PBS and resuspended for agglutination tests.

Periodate treatment: Cell suspensions were treated with sodium periodate (Sigma Chemical Co., St. Louis, Missouri, USA) at a final concentration of 0.01 *M* in saline for 1 h at 37° C. This was then centrifuged at 7000 *g* for 30 min, washed twice in PBS, and resuspended for agglutination tests.

Inhibition of agglutination: Glucose, β-D-galactose, glucosamine, galactosamine, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, L-fucose and Lactose were obtained from E. Merck, Darmstadt and Sigma Chemical Co., St Louis, Missouri, USA. Stock solutions of carbohydrates (1, 2 and 3%) were prepared in saline. Equal volumes of carbohydrate and lectin solutions were mixed and incubated at 37° C for 25 min. One drop of this mixture, was added to one drop of cell suspension, mixed, incubated and then observed under a light microscope.

Fluorescent labelling of lectins: Fluorescent labelling was carried out by the modified method of Clausen (1974) using fluorescein isothiocyanate (FITC) (Sigma Chemical Co., St. Louis, Missouri, USA). Agglutination of *Agrobacterium tumefaciens* cells by fluorescein isothiocyanate (FITC) labelled lectins were observed in a fluorescence microscope (Carl Zeiss, Jena, fluorescence microscope, NU-2 using an Osram HBO-200 light source and transmitted dark field tungsten illumination (Zeiss no. OG1 was the barrier filter while the excitor filter was B 223 g).

Tumour inhibition studies: Young healthy plants (*Bryophyllum calycinum* Salisb) with 2 to 4 internodes and stem of 5 cm diameter at the base were selected. The chosen sites on the stem were cleaned with distilled water and ethanol. Wounds were made with sterile needle by puncturing the stem. For inhibition studies the wounded areas were treated with the respective carbohydrate solutions according to the following protocol (table 1).

Table 1. Protocol for studying tumour inhibition in plants.

Carbohydrate used individually	Concentrations used in each case	Period of treatment	No. of plants per group
N-Acetyl-D-galactosamine, β-D-Galactose	1.0 mg/ml	3 h	3
Lactose, N-acetyl-D-glucosamine	2.5 mg/ml	6 h	
Galactosamine	5.0 mg/ml	24 h	
Glucosamine		36 h	
Glucose			
NaCl control	0.9%	36 h	3

For treatment, the wounded areas were covered with sterile gauze and carbohydrate solutions were applied by means of capillary tubes for the scheduled period of time. After the carbohydrate treatment, bacteria were introduced into these wound areas at a concentration of 10^8 cells/ml by means of a looped needle. The wounds were then covered with sterile cotton and the plants kept for three days at 25° C under alternate light (14 h) and dark (10 h) conditions. After this phase the cotton wrappings were removed and the plants were kept at room temperature (30° C). For control experiments, instead of carbohydrate treatment, 0.9% (w/v) NaCl was applied at wound sites. The rest of the procedure for tumour induction was the same as for the carbohydrate treated plants. Pea sized tumours (in controls) appeared after 15 days.

Results

The *Agrobacterium tumefaciens* cells were agglutinated by only two of the lectins used *viz.* lectins of *Dolichos biflorus* and *Ricinus communis*, indicating that the exposed carbohydrate residues are N-acetyl-D-galactosamine and β-D-galactose. The agglutination patterns, titers and results of the inhibition experiments are summarized in table 2. The sensitivity of the agglutination reactions towards sodium periodate, indicates that carbohydrate structures are the lectin binding determinants. Pronase treatment did not influence agglutination, showing thereby that the carbohydrate residues are not attached to proteins but to lipids as LPS on the cell surface. Fluorescence microscopy using FITC labelled lectins of *Dolichos biflorus* and *Ricinus communis* confirmed agglutination of *A. tumefaciens* by these lectins. The results of tumour inhibition studies as presented in table 3 indicate that N-acetyl-D-galactosamine and β-D-galactose do indeed participate in adherence by their ability to inhibit tumour induction.

Table 2. Agglutination of *Agrobacterium tumefaciens*, *N R* and *S* strains by the lectins.

Agrobacterium tumefaciens strains		Agglutination by lectins[a]			
		L. culinaris	*D. biflorus*[c]	*U. europaeus*	*R. communis*[d]
N :	intact cells	0	++ (32)[b]	0	++ (64)
	pronase treated	0	++	0	++
	periodate treated	0	0	0	0
R :	intact cells	0	++ (32)	0	++ (64)
	pronase treated	0	++	0	++
	periodate treated	0	0	0	0
S :	intact cells	0	++ (32)	0	++ (64)
	pronase treated	0	++	0	++
	periodate treated	0	0	0	0

[a] Intensity of the agglutination was recorded as: no agglutination O; strong agglutination ++.

[b] Titer given in parentheses is defined as the reciprocal of the highest dilution of lectins causing agglutination visible in a light microscope.

[c] Agglutination was inhibited by β-D-galactose (1%), lactose (2%), N-acetyl-D-galactosamine (1%), galactosamine (2%).

[d] Agglutination was inhibited by β-D-galactose (1%), N-acetyl-D-galactosamine (1%), galactosamine (2%), glucosamine (5%).

Discussion

Agglutination of both tumourigenic and nontumourigenic strains of *A. tumefaciens* by lectins of *D. biflorus* and *R. communis* indicates that the carbohydrate residues exposed on the cell surfaces are the same in all these strains. These results experimentally verify the observations of Lippincott and Lippincott (1969) that both

Table 3. Ability of different carbohydrates to inhibit crown gall tumour induction.

Carbohydrate used	Concentration in mg/ml	Ability to inhibit tumour induction after 3 h	6 h	24 h	36 h
N-Acetyl-D-galactosamine	1.0[a]	+	+	+	+
β-D-Galactose	1.0[a]	+	+	+	+
N-Acetyl glucosamine	5.0[b]	–	–	–	–
Glucosamine	5.0[b]	–	–	–	–
Galactosamine	5.0[b]	–	–	–	–
Glucose	5.0[b]	–	–	–	–
Lactose	5.0[b]	–	–	–	–
Control NaCl	0.9%	–	–	–	–

[a] These represent the minimum concentrations required for tumour inhibition.

[b] These represent the highest concentrations used.

strains can bind to plant cell walls. The carbohydrate residues exposed on the *Agrobacterium* cell surface *viz.* N-acetyl-D-galactosamine and β-D-galactose probably comprise the *O*-antigenic region of the LPS molecules on the surface. The *O*-antigenic region of LPS of gram-negative bacteria are primarily responsible for the serological specificity of the organisms (Wilkinson, 1977) and thus it may be reasonable to assume that these exposed carbohydrates might play a major role in the adherence of *Agrobacterium* to the plant cells. This is in agreement with a previous report from this laboratory that N-acetyl-D-galactosamine and β-D-galactose are involved in adherence during crown gall tumour initiation (Banerjee *et al.*, 1981). However, the ultimate proof that these carbohydrate residues do indeed participate in adherence must come from *in vivo* studies. Results obtained from tumour inhibition studies using various concentrations of carbohydrates for different time period (table 3) demonstrate that N-acetyl-D-galactosamine and β-D-galactose take a positive role in host-bacterium adherence during crown gall tumour initiation.

The minimum time required for tumour inhibition by N-acetyl-D-galactosamine and β-D-galactose at concentrations of 1 mg/ml was 3 h. Lippincott and Lippincott (1980) had reported that cell wall preparations of virulent and avirulent *Agrobacterium* strains could inhibit tumour induction when added only 15 min prior to infection of wound sites of host plants with virulent strains of *Agrobacterium*. Such a short time period for tumour inhibition may be explained by considering that carbohydrate residues when present as part of the bacterial cell wall preparations are afforded a better anchorage than when present free in solution. Thus, free N-acetyl-D-galactosamine and β-D-galactose require a longer period of time to saturate and mask the available binding sites of the host plant cell wall, thereby rendering them inaccessible for further attachment.

On the part of the host, polygalacturonic acid molecules located in outermost region of the plant cell wall and probably exposed by wounding have been shown to participate in the adherence process (Lippincott and Lippincott, 1980).

The mechanism of interaction of N-acetyl-D-galactosamine and β-D-galactose of the *Agrobacterium* cell surface with the plant cell wall polygalacturonic acid molecules is not clear at present and it may be assumed that the interaction takes place either involving van der Wall's forces or by enzymes leading to chemical bonding of the interacting molecules.

Currently we are engaged in studying the physical and chemical properties of *Agrobacterium tumefaciens* LPS in order to get a better idea of its structure and biological function. Preliminary studies indicate that LPS of *Agrobacterium* are complex molecules comprised of polysaccharide side chain linked to lipid *A via.* a core region containing mainly KDO (2 keto 3 deoxy octulosonate) and orthophosphate. Total carbohydrate content is 20.2%. Fatty acid analyses reveal that hydroxylated fatty acids comprise nearly 60% of the total fatty acids. Physical data indicate that the size of the aggregated particle is in millions of daltons. However, the disaggregated molecular weight worked out to be 250,000 daltons in the analytical ultracentrifuge. Electron microscopy of the LPS by negative staining revealed large vesicle like structures very similar to other bacterial LPS. A protein moiety identified in our LPS preparation suggests, that it is associated with LPS in the native state. Amino acid analysis reveals that it is not an artifact.

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The use of concanavalin A in the purification or separation of multiple forms of brain hydrolases

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Abstract. Concanavalin A bound to Sepharose has been used for the purification of brain β-galactosidase, α-L-fucosidase, α-D-mannosidase, arylsulphatase and β-glucuronidase. Several factors *viz* pH, temperature and concentration of α-methyl glucoside influenced the binding and elution of these enzymes. A lysosomal acid α-mannosidase and a cytosolic neutral mannosidase were separable by concanavalin A-Sepharose chromatography. Similarly lysosomal and microsomal β-glucuronidases were separable using gradient elution with α-methyl glucoside. The results indicate the usefulness of this lectin for the isolation of wide variety of enzymes under specified experimental conditions.

Keywords. Concanavalin A; chromatography; brain hydrolases.

Introduction

Concanavalin A (Con A), a lectin derived from *Canavalia ensiformis* or *Canavalia gladiata* has found widespread use in the purification of several glycoproteins. It has a specificity towards binding α-D-mannopyranoside or α-D-glucopyranoside structures containing unmodified C-3, C-4 and C-6 hydroxyl groups (Goldstein, 1976). An earlier observation made in this laboratory by Bishayee and Bachhawat (1974) indicated that several lysosomal enzymes of the brain tissue bind to Con A linked to Sepharose-4B. Following these observations, we found that Con A-Sepharose could be conveniently used for the purification or separation of multiple forms of brain lysosomal enzymes. In the present study we report our observations on the separation by affinity chromatographic using Con A-Sepharose of β-D-galactosidase, α-L-fucosidase, α-D-mannosidase, arylsulphatase and β-D-glucuronidase of monkey brain.

Materials and methods

Con A was prepared from *C. gladiata* according to Surolia *et al.* (1973), and coupled to cyanogen bromide activated Sepharose 4-B as described by Cuatrecasas and Parikh (1972). The usual concentration of Con A bound to Sepharose was about 10 to 15 mg/ml gel. α-Methylglucoside and the *p*-nitrophenyl derivatives of the various glycosides, used as substrates, were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Nitrocatechol sulphate was prepared according to Dodgson and Spencer (1957).

β-D-Galactosidase, α-L-fucosidase, α-D-mannosidase, arylsulphatase and β-D- glucuronidase were assayed as described earlier (Alam and Balasubramanian, 1978a, b; Lakshmi and Balasubramanian, 1980; Mathur and Balasubramanian, 1981; Alvares and Balasubramanian, 1982.

Protein was estimated according to Lowry *et al.* (1951).

Results

The experimental condition facilitating the binding to Con A-Sepharose and elution of the acid-glycosidases of monkey brain are summarised in table 1. The pH of binding, the pH of elution, the concentration of α-methylglucoside used for elution, and the temperature of elution affected the chromatography of these enzymes on the Con A-Sepharose.

Table 1. Experimental conditions for the binding and elution of brain enzymes to Con A-Sepharose.

Brain enzyme	Binding characteristics to Con A-Sepharose	Elution with α-methyl glucoside
β-D-Galactosidase	Binds at pH 6.0. Both galactosidases *A* and *B* bind	Elutable at 25°C. No significant elution at 4°C.
α-L-Fucosidase	Binds at pH 6.0. All the three forms of fucosidase bind	Elutable at 25°C
α-D-Mannosidase	The cytosolic neutral mannosidase does not bind but lysosomal acid mannosidase binds at pH 6.0	Elutable at 25°C
β-D-Glucuronidase	Both lysosomal and microsomal glucuronidases bind at pH 6.0	Both enzymes are eluted at 28°C. The lysosomal enzyme is eluted at low and the microsomal enzyme at high concentration of α-methyl glucoside

β-*Galactosidase*

β-D-Galactosidase was purified from monkey brain homogenate through five steps (Alam and Balasubramanian, 1978a), and a key step in the purification of the enzyme was Con A-Sepharose affinity chromatography. The enzyme eluted from this column upon subsequent Sepharose-6B gel filtration, resolved into a high molecular weight (β-galactosidase *B*), and a low molecular weight (β-galactosidase *A*) component. Galactosidase *B* was purified to apparent homogeniety as evidenced by sodium dodecyl sulphate (SDS) gel electrophoresis (Alam and Balasubramanian, 1978a). The *B* enzyme had a molecular weight of approximately 1.2×10^6 and the *A* enzyme about 1.2×10^5, ten times lesser than *B*. Both the enzymes were similar in their pH optima, K_m value for *p*-nitrophenyl β-galactoside and competitive inhibition by γ-D-galactonolactone. The specificity of the monkey brain β-galactosidases towards glycolipid substrates galactosylceramide, lactosylceramide and GMI ganglioside were somewhat similar to the findings of Tanaka and Suzuki (1977).

α-L-Fucosidase

An ammonium sulphate fraction of this enzyme from monkey brain could be purified about 170 fold in a single step by Con A-Sepharose chromatography (Alam and Balasubramanian, 1978b). The enzyme was bound at pH 6.0 to the Con A-Sepharose and eluted with α-methyl glucoside at 25° C. The purified enzyme on Sephadex G-200 gel filtration gave evidence for three peaks of enzyme activity. These three enzyme activities differed not only in their molecular weight, but also in their pH optima and thermal stability (Alam and Balasubramanian, 1978b).

α-D-Mannosidase

This enzyme was shown to be present in the monkey brain in two forms, an acid mannosidase present in the lysosomal fraction and a neutral mannosidase present in the cytosol. These two enzymes could be conveniently separated from each other by Con A-Sepharose chromatography (Mathur and Balasubramanian, 1981). While the acid mannosidase totally bound to the column (and eluted by α-methyl glucoside) the neutral enzyme did not bind to the column. It is presumable that the difference in carbohydrate composition of the two forms of enzymes might be responsible for their behaviour. The acid and neutral mannosidases were found to differ also in their response to metal ion activation, pH optima, thermal stability and molecular weight (Mathur and Balasubramanian, 1981).

Arylsulphatases

The arylsulphatase *A*, *B* and B_m of brain could be separated from one another by DEAE-cellulose column chromatography (Lakshmi and Balasubramanian, 1980). All these enzymes bound to Con A-Sepharose could be eluted with α-methyl glucoside. An unusual property exhibited by the arylsulphatase B_m was, that after Con A-Sepharose chromatography, it was totally and irreversibly bound by Sephadex G-200 (Lakshmi and Balasubramanian, 1980).

β-D-Glucuronidases

The monkey brain contained a lysosomal and a microsomal glucuronidase as observed in other tissues (Tulsiani *et al.*, 1978). They had similar pH optima and both of them bound to Con A-Sepharose. They could be partially separated from each other by using gradient elution with α-methyl glucoside. It is possible that the two glucuronidases differ in their glucose or mannose content resulting in their differential elution pattern (Alvares and Balasubramanian, 1982).

Discussion

The above results indicate that Con A bound to Sepharose can be most conveniently used not only for the isolation and purification of the lysosomal acid glycosidases of the brain, but also for the separation of the non-lysosomal and neutral glycosidases from the acid glycosidases. Use of this lectin-Sepharose has also been found in getting a concentrated form of the lysosomal enzymes from body fluids such as cerebrospinal fluid (Cherian and Balasubramanian, 1978). In summary Con A-Sepharose can be used;(a) for the purification of the lysosomal enzymes,

(b) for getting a concentrated form of these enzymes from body fluids, and (c) for a preliminary assessment of the carbohydrate composition of these enzymes.

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Lectins from *Griffonia simplicifolia* seeds*

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Abstract. The physical-chemical and carbohydrate binding specificity of *Griffonia simplicifolia* I (GS I) isolectins, one of the 4 lectins isolated from *Griffonia simplicifolia* seeds, are described.

Association constants for the binding of methyl α- and β-D-galactopyranoside and methyl 2-acetamido-2-deoxy-α-D-galactopyranoside to the A_4, A_2B_2 and B_4 isolectins are reported.

Precipitation reactions of the *Griffonia simplicifolia* isolectins with guaran and type B blood group substance are described.

The hypothesis that subunit B is a precursor of subunit A, a process involving proteolytic cleavage of the B subunit, was tested by conducting structural studies on the 2 subunits. The results indicated that the A and B subunits are probably products of 2 separate but closely related, possibly contiguous genes.

Keywords. Lectin; *Bandeiraea simplicifolia*; *Griffonia simplicifolia*; isolectins; carbohydrate binding.

Introduction

Griffonia simplicifolia, formerly called *Bandeiraea simplicifolia*, is a woody shrub which grows in the rain forests of Northwest Africa. It is especially abundant in Ghana. The mature plant reaches a height of 3–4 metres and develops pods which explode with great force dispersing the flat, round black seeds. Thus far we have identified and isolated four distinctly different lectins from *G. simplicifolia* seeds:

GS I—A system of 5 α-D-galactosyl binding isolectins
GS II—An N-acetyl-D-glucosaminyl binding lectin
GS III—A system of 5 2-acetamido-2-deoxy-α-D-galactosyl binding isolectins
GS IV—A lectin with Le^b (Lewis b) binding properties

This report will focus on the GS I isolectins.

Results

Mäkela and Mäkela (1956) first reported a human anti-B haemagglutinating activity in *G. simplicifolia* seeds. Employing an affinity matrix prepared by copolymerization of acrylamide with allyl α-D-galactoside, Horęjsi and Kocourek (1973)

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Abbreviations used: GS I, A system of 5α-D-galactosyl binding isolectins; GS II, a N-acetyl-D-glucosaminyl binding lectin, GS III, a system of 5,2-acetamido-2-deoxy-α- D-galactosyl binding lectins; GS IV, a lectin with Le^b (Lewis-b) binding properties, all lectins isolated from *G. simplicifolia* seeds; PAGE, polyacrylamide gel electrophoresis; SDS-PAGE, sodium dodecylsulphate PAGE; BSA, bovine serum alubmin; p-NO_2- ph, p-nitrophenyl.

purified the anti-blood group B lectin but did not characterize it. Subsequently, Hayes and Goldstein (1974) obtained what they believed to be a homogeneous glycoprotein by affinity chromatography on a melibionate Bio-Gel column. The glycoprotein gave one band on polyacrylamide gel electrophoresis (PAGE) at pH 4.3, one band on sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) at pH 7.0, a single symmetrical peak in the ultra-centrifuge and was quantitatively precipitated by the galactomannan guaran. However, multiple bands were observed on PAGE at pH 9.5 and on isoelectric focusing. A subunit molecular weight of 31,000 was calculated from gel electrophoresis, and 28,500 from the amino acid and carbohydrate composition. The intact lectin is composed of 4 subunits and has a molecular weight of approximately 114,000, as determined by sedimentation velocity centrifugation and gel filtration. A metalloprotein, the lectin requires Ca^{2+} for maximum activity. Analysis of the lectin by circular dichroism spectroscopy indicated that the native lectin contains a large proportion (30–40%) of β-structure (Lonngren *et al*, 1976).

Three experimental findings suggested that the GS I lectin was not a single homogeneous molecular species: 5 bands by alkaline PAGE, less than stoichiometric amounts (*ca* 0.5) of methionine/subunit, and non-sigmoidal inhibition curves generated, when N-acetyl-D-galactosamine and its derivatives were assayed for their ability to inhibit the GS I — guaran precipitation reaction (Hayes and Goldstein, 1974; Murphy and Goldstein, 1977, 1979).

Indeed, GSI. plant seed isolectins comprise a family of tetrameric α-D-galactopyranosyl-binding glycoproteins, composed of various combinations of two different kinds of subunits designated A and B. Subtypes of the A and B subunits have been demonstrated by isoelectric focusing (Murphy and Goldstein, 1979).

Advantage is taken of the different carbohydrate binding specificity of the two types of subunits in order to resolve the mixture. The A subunit exhibits a primary specificity for N-acetyl-α-D-galactosaminyl groups, but also cross-reacts with α-D-galactosyl groups, whereas the β-subunit shows a rather sharp specificity for α- D-galactosyl units. The isolectins, designated A_4, A_3B, A_2B_2, AB_3 and B_4, all bind to a melibionate-Bio Gel column. The A_4 and A_3B isolectins were displaced from the melibionate-column as purified components by increasing quantities of N- acetyl-D-galactosamine (Murphy and Goldstein, 1977). The A_2B_2, AB_3 and B_4 components after elution from the melibionate column with methyl α-D-galactoside, were resolved on a column of insolubilized blood group A substance. The latter affinity column has N-acetyl-α-D-galactosaminyl determinant groups which interact with the binding sites of the A subunit. A more recent procedure allows separation of all the 5 isolectins on a single affinity column which takes advantage of the differential interaction of the A and B subunits with a *p*-aminophenyl β-D-galactopyranoside-derivatized column matrix (Delmotte and Goldstein, 1980).

Physico-chemical characterization of the A_4 and B_4 isolectins reveals the A and B subunits to be closely related glycoproteins. The carbohydrate composition of the two subunits is identical; determined to the nearest integer, both contain N-acetyl glucosamine (2 mol), mannose (8 mol), xylose (2 mol), fucose (2 mol) and traces of arabinose, glucose and galactose (Murphy and Goldstein, 1979). The amino acid composition of the A and B subunits (table 1) are very similar suggest-

Table 1. Amino acid composition of *G. simplicifolia* A_4 and B_4 isolectins[a]

	A_4	B_4
Lys	12	9
His	2	2
Ammonia	29	35
Arg	7	7
Asp	36	33
Thr	20	21
Ser	30	31
Glu	14	19
Pro	12	13
Gly	16	15
Ala	19	19
1/2-Cys[b]	1	1
Val	18	19
Met	0	1
Ile	13	11
Leu	21	19
Tyr	10	9
Phe	16	16
Trp[c]	8	7

[a] Data are expressed in terms of amino acid residues (nearest integer) per subunit.

[b] Determined by hydrolysis in the presence of dimethyl sulphoxide.

[c] Determined spectrophotometrically.

ing that they are homologous structures; in fact rabbit antisera to the A_4 isolectin cross reacts extensively with the B_4 isolectin and *vice versa.* There is one notable difference between the two subunits: the B subunit has one methionine residue whereas the A subunit contains no methionine.

A large number of precipitin reactions of *G. simplicifolia* I isolectins A_4 and B_4 with naturally occurring biopolymers and carbohydrate-bovine serum conjugates have been carried out (Murphy and Goldstein, 1977, 1979; Wood *et al.*, 1979). Both A_4 and B_4 formed precipitates with biopolymers containing multiple, nonreducing α-D-Gal*p* end groups; these include guaran (a galactomannan) and type B blood group substance. Biopolymers containing multiple, nonreducing β-D-Gal*p* end groups such as larch arabinogalactan, did not form precipitates with either isolectin (Murphy and Goldstein, 1979).

A_4 precipitated well with blood groups A_1, A_2, B and precursor substances, with A_2 precipitating less strongly than did A_1 substance; H, Le^a and Le^b substances did not react (Wood *et al.*, 1979). Precipitin inhibition and competitive binding assays confirmed the precipitin data that A_4 is most specific for terminal nonreducing α-linked 2-acetamido-2-deoxy-D-galactopyranose, but also reacts with oligosaccharides with terminal non-reducing α-linked-D-galactosyl end groups (Wood *et al.*, 1979). B_4 precipitated well with B blood group substances and with a precursor substance to a lesser extent, but did not react with A_1 A_2, H, Le^a or Le^b substances (Wood *et al.*, 1979).

Interestingly, a *p*-azophenyl α-D-galactopyranoside-bovine serum albumin (BSA) conjugate did not precipitate with either A_4 or B_4, whereas *p*-azophenyl β-D-galactopyranoside-BSA reacted very weakly with A_4 and strongly with B_4. The *p*-azophenyl β-lactoside-BSA conjugate did not form a precipitate with either A_4 or B_4. A number of biopolymers containing multiple, nonreducing α-D-GalNAc*p* end groups were also tested for their ability to form precipitates with A_4 and B_4. Type A blood group substance, a streptococcus type C cell wall polysaccharide, and asialo bovine submaxillary mucin precipitated strongly with A_4, whereas only asialo bovine submaxillary mucin precipitated a small amount of B_4. Native bovine submaxillary mucin which probably contains a smaller number of incomplete carbohydrate chains with exposed terminal, nonreducing α-D-GalNac*p* residues reacted weakly with A_4 but not with B_4. A_4 precipitated twice as much *p*-azophenyl-N-acetyl-α- D-galactosamine-BSA as the β anomer (*p*-azophenyl-N-acetyl-β-D-galactosamine-BSA) whereas B_4 did not precipitate either of these conjugates.

The best inhibitors of the *B. simplicifolia* I isolectin B_4-guaran system were Me-α-D-Gal*p*, *p*-nitrophenyl (*p*-NO_2Ph)-α-D-Gal*p*, and *p*-NO_2Ph-β-D-Gal*p*. Comparison of Me-α-D-Gal*p* with Me-β-D-Gal*p* and a series of oligosaccharides containing nonreducing α-and β-D-galactopyranosyl end units, reveals that the α-anomer is bound more strongly than the corresponding β-anomer. Comparison of Me-α-D-Gal*p*, Me-β-D-Gal*p*, and D-galactose supports the conclusion that a methoxyl group in the α position contributes positively to stabilization of the B_4 sugar complex, whereas a β-methoxyl group represents a destabilising factor. Substitution of the oxygen atom at the C-1 position of methyl β-D-galactoside by a sulphur atom (methyl β-D-thiogalactoside) produced a 3.5-fold decrease in the inhibiting potency of this glycoside (Murphy and Goldstein, 1979).

The interaction of the *p*-nitrophenyl α- and β-D-galactosides with B_4 is remarkable. In sharp contrast to the results obtained with the α- and β-galactosides with aliphatic aglycons, *p*-nitrophenyl α- and β-D-galactoside were equally inhibitory. In as much as methyl and *p*-nitrophenyl α-D-galactoside were equally potent as inhibitors, it is apparent that the aromatic aglycone does not enhance the capacity of the galactoside to bind to the lectin. However, *p*-NO_2Ph-β-D-Gal*p* was 6 times more potent an inhibitor than Me-β-D-Gal*p*, indicating a strong enhancement of binding by the aromatic aglycon of this β-D-galactoside. This finding formed the basis of a new procedure for the separation of the GS I isolectins (Delmotte and Goldstein, 1980).

The *B. simplicifolia* I A_4 isolectin also has a preference for the α-anomeric configuration: α-GalNac*p* groups are bound more strongly than the anomeric β-glycosides. It was also observed that the *p*-nitrophenyl α- and β-D-glycosides of D-GalNAc are 2–4 times better inhibitors than the corresponding methyl glycosides of the same anomeric configuration. These results indicate a role of aromatic aglycons in the binding mechanism of glycosides to the A_4 isolectin.

The disaccharides 3-*O*-(α-D-GalNAc*p*)-D-Gal, 6-*O*-(α-D-GalNac*p*)-D-Gal, and 3-*O*-(α-D-GalNAc*p*)-D-Glc were all excellent inhibitors in the A_4-guaran system, being 7–25% better inhibitors than methyl α-D-GalNAc*p*.

The 2-acetamido-2-deoxy group appears to represent an important binding locus for the A_4-D-GalNAc*p* interaction. The observation that D-GalNAc is 13 times more inhibitory than D-Gal, and 72 times better than 2-deoxy-D-galactose supports this view. Furthermore, Me-β-D-GalNAc*p* is 55 times more potent an inhibitor than methyl 2-amino-2-deoxy-α-D-galactopyranoside.

Using deoxy and fluoro galactose derivatives it has been possible to determine whether the *H* or *O* atom of the hydroxyl groups are involved in hydrogen bonding to the lectin. The fact that neither methyl 4-deoxy-4-fluoro-α-D-galactopyranoside (20 mM) nor *p*-nitrophenyl 3-deoxy-3-fluoro-β-D-galactopyranoside (10 mM), inhibited the A_4- or B_4- guaran interaction suggests that the *hydrogen* atom and not the oxygen atoms of the C-3 and C-4 hydroxyl groups are involved in hydrogen bonding to the lectin.

Association constants for the binding of methyl α-D-galactopyranoside (methyl α-D-Gal*p*) and methyl 2-acetamido-2-deoxy-α-D-galactopyranoside (methyl α-D-GalNAc*p*) to three *Griffonia simplicifolia* isolectins (A_4, A_2B_2, B_4) were determined by equilibrium dialysis and fluorescence enhancement measurements. The A and B subunits appear to have approximately the same K_{assoc} for methyl α-D-Gal*p*: 1.45×10^4, 1.98×10^4, and 2.06×10^4 M^{-1} for A_4, A_2B_2, and B_4, respectively, as determined by equilibrium dialysis (Goldstein *et al.*, 1981). Fluorescence enhancement measurements on B_4 gave an association constant of 2.07×10^4 M^{-1} for methyl α-D-Gal*p* and 1.87×10^3 M^{-1} for methyl β-D-Gal*p*. By equilibrium dialysis, we were able to detect 3.3 (theory, 4.0) methyl α-D-GalNac*p* binding sites for A_4 ($K_{assoc} = 1.87 \times 10^5$ M^{-1}), 1.9 for A_2B_2 ($K_{assoc} = 1.19 \times 10^5$ M^{-1}), and were unable to detect any methyl α-D-GalNac*p* binding sites for B_4 (Goldstein *et al.*, 1981). However, four very weak methyl α-D-GalNac binding sites for B_4 were detected by fluorescence enhancement measurement ($K_{assoc} = 1.26 \times 10^2$ M^{-1}). Thus, the A subunit has an affinity for methyl α-D-GalNac 3 orders of magnitude greater than the B subunit (Goldstein *et al.*, 1981).

Using 4-methylumbelliferyl glycosides, DeBoeck and his colleagues measured binding characterstics of α-D-galactosyl, β-D-galactosyl and 2-acetamido-2-deoxy-β-D-galactosyl groups to A_4 and B_4 (DeBoeck *et al.*, 1981).

Precipitation and hapten inhibition data are in accord with these binding measurements. Toward guaran and type B blood group substance which contain α-D-Gal*p* end groups, all isolectins precipitated the same amount of biopolymer. However, AB_3, A_2B_2, and A_3B, which are mono-, di-, and trivalent for α-D-GalNAc*p*, were differentially precipitated by type A blood group substance which contains α-D-GalNAc*p*-end groups. A_3B precipitated the most, A_2B_2 less, and AB_3, no type A substance (figure 1). These isolectins are being studied in order to evaluate the effect of valence on lectin-cell interaction.

Interestingly, the amount of each individual GS I isolectin varies from seed to seed, each seed displaying an unique distribution of the 5 isolectins (Murphy and Goldstein,1979; Lamb *et al.*, 1981). In fact, no two seeds are exactly alike in their isolectin content. Some seeds contain only B_4 or A_4; others have only A_4, A_3B and A_2B_2; still others contain all the five isolectins (Murphy and Goldstein, 1979; Lamb *et al.*, 1981).

Two possible mechanisms could explain the formation of the five isolectins: the A and B subunits could simply combine randomly following polypeptide transla-

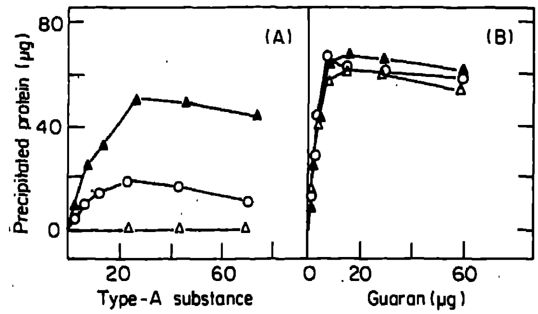

Figure 1. Quantitative precipitation reactions of *B. simplicifolia* I isolectins with glycoconjugates. **A.** Interaction of A_3B (61 μg) (▲), A_2B_2 (70 μg) (○), and AB_3 (61 μg) (△), with varying amounts of ovarian cyst blood type A substance. **B.** Interaction of A_3B (61 μg) (▲), A_2B_2 (70 μg) (○), and AB_3 (61 μg) (△), with varying amounts of guaran. (Published with permission of *J. Biol. Chem.*).

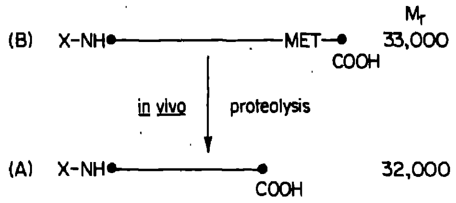

Figure 2. Model showing proposed proteolytic conversion of BS-I B subunit to A subunit. X — pyroglutamate groups.

tion and processing, *e.g.* according to a binomial distribution based on the number of A and B subunits in each isolectin; or a precursor-product relationship may be involved in which proteolytic cleavage of the intact tetrameric B_4 isolectin gives rise sequentially to the remaining four isolectins. A feasible model incorporating these considerations is shown in figure 2.

Alkaline, nondenaturing polyacrylamide gel electrophoresis was employed to analyze the distribution of the five isolectins from 168 single seeds gathered in Ghana from known trees and pods (Lamb *et al.*, 1981). The gels were scanned at 625 nm and the peak height corresponding to each isolectin was measured and expressed as a percentage of the total peak height. Principal components analysis of the correlations among the percentages indicated a sequential relationship among the five isolectins with the concentrations of B_4 and A_4 inversely related. Further statistical analyses indicated that although the distribution of isolectins in each seed was unique, seeds gathered from the same tree were more similar than seeds gathered from different trees.

The existence of a precursor — product relationship allows several predictions with regard to the structure of the A and B subunits (figure 2): (i) the N-terminus of both subunits should be identical; (ii) the *C*-terminus of the subunits should differ; and (iii) CNBr-cleavage of the B subunit should yield a small polypeptide and a large piece, identical to the A subunit.

To test these predictions we have carried out chemical studies on the A and B subunits and the preliminary results are presented here.

Carboxypeptidase Y digestion of the A and B subunits gave identical results, the last six *C*-terminal amino acids being: Ser-Thr-Leu-(Phe, Val)-Arg-COOH. Both subunits were blocked at their *N*-terminus by pyroglutamate groups. Removal of these blocking groups with calf-liver pyroglutamate permitted determination of the first 25 *N*-terminal amino acids by automated Edman degradation. There were four differences between the A and B subunits.

Cleavage of the B subunit with CNBr gave a large and a small piece (30,000 and 3,000 daltons, respectively). The large piece was assigned to the *C*-terminus and the small fragment to the *N*-terminus of the B subunit. By SDS-PAGE analysis, the large piece was smaller than the A chain.

Analysis of tryptic peptides by reverse-phase HPLC showed that approximately 30% of the tryptic peptides were common to both the A and B subunits; each subunits also contained unique peptides. The accumulated evidence indicates that the precursor-product relationship is invalid. On the basis of the present studies, it is proposed that the A and B subunits are products of two separate but closely related, possibly contiguous genes.

Labelled with fluorescein, ferritin, colloidal gold and tritium the GS I-B_4 isolectin has been used as a probe for the detection of α-D-galactosyl groups in plant and animal cells. In its immobilized form, the B_4 isolectin was used to isolate the galactomannan from *Cassia alata* seeds (Ross *et al.*, 1976). The B_4 isolectin agglutinates Ehrlich ascites tumour cells and a family of α-D-galactosyl-containing glycoproteins was isolated from an Ehrlich cell membrane fraction (Eckhardt and Goldstein, 1979). Fluoresceinated B_4 was shown to bind to murine kidney cortex sections and we discovered that this α-D-galactosyl binding isolectin could serve as a specific probe for basement membrane of the mouse, rat, rabbit and cow (Peters and Goldstein, 1979).

The GS I-B_4 isolectin has also been shown to be cytotoxic to a variety of animal cell; it was used to generate a variant clone of B_4-resistant 3*T*3 cells (Stanley *et al.*, 1979).

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The effect of tumour progression on lymphocyte responses to lectins, measured by fluorescence polarization techniques

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Abstract. The process of fluorochromasia involves the hydrolysis by cells of fluorescein diacetate resulting in an intracellular accumulation of fluorescein. The polarization of the fluorescence of the fluorescein appears to depend on the intracellular fluorescein concentration, the distribution of fluorescein within the cell and the viscosity of the cell cytoplasm.

The parameters of fluorochromasia were studied with thymocytes from normal BALB/c mice and from mice bearing an intraperitoneal NK/LY/R lymphoma. During the course of tumour proliferation, the response to *T*-cell mitogens increased whereas the response to other lectins, *e.g.* wheat germ agglutinin, decreased or remained unaltered. These changes were consistent with the corresponding increase in immunocompetent cells within the thymus, observed by microelectrophoresis. Thus this sensitive technique provides a useful quantitative assessment of the lectin-lymphoid cell interaction.

Keywords. Fluorescein; polarization; lymphocytes; fluorescein fluorescence polarization; fluorochromasia.

Introduction

Fluorochromasia in lymphoid cells, first described by Rotman and Papermaster (1966), involves the intracellular hydrolysis of the non-fluorescent substrate fluorescein diacetate (FDA) to which the cell membrane is permeable forming free fluorescence fluorescein. Since the membrane is less permeable to the reaction product, the net result is an intracellular accumulation of fluorescein increasing with the duration of the reaction. Measurement of fluorescence polarization during fluorochromasia was first carried out by Burns (1969), using yeast cells of *Euglena gracillis* cells. The microviscosity of the cell interior was estimated, and the effects of phenethyl alcohol on yeast cells were determined(Burns, 1971). Cercek and Cercek (1972a, b; 1973) also measured fluorescein fluorescence polarization in yeast cells, subsequently using the technique to make similar measurements on Chinese Hamster ovary cells (Cercek *et al*, 1973) and human lymphocytes (Cercek *et al*, 1974). They were able to measure a decrease in the polarization of fluorescence caused by *in vitro* incubation of human peripheral blood lympho-

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Abbreviations used: FDA, Fluorescein diacetate; WGA, wheat germ agglutinin; Con A, concanavalin A; PBS, 0.15M phosphate buffer, pH 7.3 containing 0.15 M sodium chloride; PWD, Poke Weed Mitogen.

cytes with phytohaemagglutin (PHA). However, no response to PHA of lymphocytes from patients with cancer could be demonstrated. The response of normal human lymphocytes to PHA as measured by fluorochromasia has been confirmed by many other groups (Dickinson *et al.*, 1976; Kreutzmann *et al.*, 1978; Pritchard and Sutherland, 1978; Nishifuku *et al.*, 1977; Takaku *et al.*, 1977; Stewart *et al.*, 1979; Balding *et al.*, 1980; Mitchell *et al.*, 1980; Orjasaeter *et al.*, 1979) other lectins, *e.g.* concanavalin A (Con A) or wheat germ agglutinin (WGA) have also been shown to alter polarization of fluorescence in lymphoid cells (Balding *et al.*, 1978, 1981). In addition, PHA induced changes in the rate of FDA hydrolysis and leakage of fluorescein from the cells could be measured (Balding *et al.*, 1980, 1981).

The level of the response to PHA of lymphocytes from patients with cancer is still controversial. A number of groups have found similar responses to those of lymphocytes from healthy donors (Dickinson *et al.*, 1976; Orjasaeter *et al.*, 1979; Balding *et al.*, 1980; Mitchell *et al.*, 1980), while others have confirmed the lack of response reported by the Cerceks (Takaku *et al.*, 1977; Kreutzmann *et al.*, 1978; Pritchard and Sutherland, 1978). A cancer-related change in the density of *T*-lymphocytes has been suggested (Pritchard *et al.*, 1978), whereas Orjasaeter *et al.* (1979) suggest that a qualitative change, unrelated to cell density, has occurred in the *T*-lymphocyte population.

In order to gain information about the relationship of changes in the *T*-lymphocyte population during the growth of a tumour and the differing response of such lymphocytes to lectins during fluorochromasia, we have used thymocytes and spleen lymphocytes from normal BALB/c mice and from mice bearing an intraperitoneal ascites lymphoma as a model system. Changes in polarization of fluorescence, rate of FDA hydrolysis and leakage of fluorescin from the cell resulting from incubation of the lymphocytes with several different lectins are reported.

Materials and methods

Preparation of lymphocyte suspensions was essentially as previously described (Balding *et al.*, 1980). Briefly, human lymphocytes from healthy donors were isolated on Lymphoprep^R (Nyegaard and Co., Oslo, Sweden, density 1.079 g/ml) at 15° C after carbonyl-iron treatment of blood samples. Murine thymocytes and spleen lymphocytes were isolated from homogenates of excised thymus or spleen tissue obtained from 6-10 weeks old healthy male inbred BALB/c mice, or from similar mice in which an intraperitoneal NK/LY/R ascites lymphoma had been cultured for the appropriate length of time. Erythrocytes in the spleen but not thymus cell suspensions were lyzed by treatment with 5 mM Tris buffer 0.83% ammonium chloride solution, pH 7.3.

Lymphocytes were washed twice in medium 199 with Earles salts, 25 mM HEPES and *L*-glutamine (Gibco Biocult) and twice in 0.15 M phosphate buffer, pH 7.3 containing 0.15 M sodium chloride (PBS) (0.45 μ Millipore filtered complete Dulbecco phosphate buffered saline, pH 7.3; from Oxoid Ltd., code SR38 plus Code BR14a). Cells were resuspended in PBS at a concentration of 8×10^6 (murine lymphocytes) or 4×10^6 (human lymphocytes). Cell suspensions were equilibrated at 37° C before addition of 0.1 ml of a saline solution of lectin [PHA: Wellcome purified grade HA16, wheat germ agglutinin (WGA), and Poke Weed Mitogen (PWM), from Sigma London Chemical Co. Ltd.] or 0.1 ml of sterile saline solution to control samples.

Fluorescence and fluorescence polarization measurements were carried out as described previously (Balding *et al.*, 1978, 1980, 1981) using a Perkin Elmer MPF4 spectrofluorimeter. The data was collected using a purpose built analogue to digital converter and analysed by computor (Data General Nova) to avoid subjective bias. Polarization of fluorescence was calculated using the simple formula developed by Perrin (1929), $p = Iv - Ih/Iv + Ih$, where Iv is the vertical component and Ih the horizontal component of intracellular fluorescence. Total fluorescence intensity Ft and extracellular fluorescence intensity Fs were calculated from the basic equation, $F = Iv + 2Ih - Fo$, where Fo is the contribution to fluorescence intensity from spontaneous hydrolysis of FDA substrate measured after an appropriate time using substrate solution without added cell suspension. Inttacellular fluorescence $Fi = Ft - Fs$.

Fluorescence and fluorescence polarization measurements were carried out at 27° C, after 30 min incubation with lectin or saline at 37° C.

Results

An immediate increase in fluorescent intensity was observed after addition of lymphocytes to FDA. The rate of hydrolysis of EDA to fluorescein by human lymphocytes was almost linear, but the rate of accumulation of intracellular fluorescein decreased to a plateau after about 14 min; the polarization of fluorescence tends towards a plateau simultaneously with the intracellular fluorescence (figure 1). During the course of the reaction there was some leakage of fluorescein out of the cells, increasing from about 10% to about 50% of the total fluorescence intensity after 20 min. Polarization of values were therefore compared at equivalent intracellular fluorescein concentration, rather than at equivalent duration of hydrolysis as is usually reported (Cercek *et al.*, 1973, 1978).

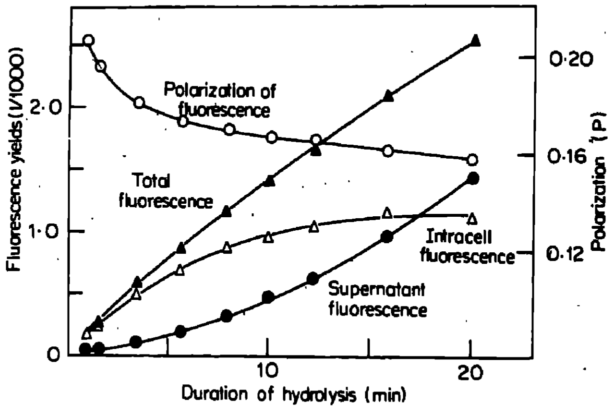

Figure 1. Fluorochromasia in human lymphocytes and the relationship between polarization of fluorescence and duration of FDA hydrolysis at 27°C (Abscissa-arbitrary units 1000 $u = 8$ nM NaF).

When lymphocytes were incubated with increasing concentrations of PHA a typical saturation curve was obtained for human lymphocytes and murine spleen lymphocytes (figure 2). Murine thymocytes from control animals, on the other hand did not show any change in polarization value on incubation with PHA.

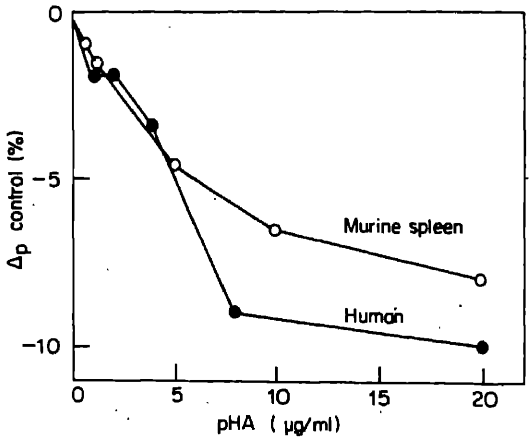

Figure 2. Change in polarization of fluorescein fluorescence for human and murine spleen lymphocytes incubated with PHA.

However, if the thymocytes were prepared from a mouse bearing a 3-day old NK/LY/R ascites lymphoma a similar saturationcurve as observed for normal spleen cells was obtained (figure 3). In contrast, thymocytes from the control animal gave a decrease in polarization value after incubation with WGA. Thymocytes from the tumour-bearer showed a much reduced response or a slight increase in polarization value after incubation with WGA.

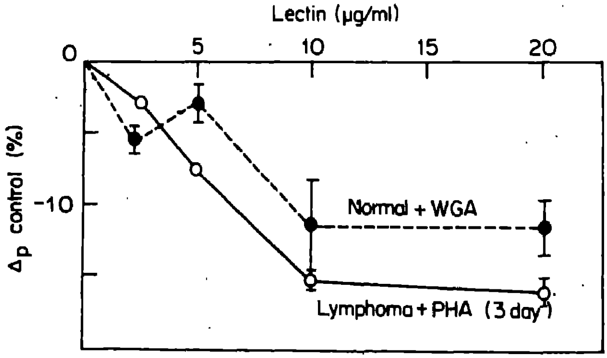

Figure 3. Change in polarization of fluorescein fluorescence for murine thymocytes from control andNK/LY/Rlymphoma bearing animals, incubated with WGA or PHA (± s.e.).

A similar decrease in polarization of fluorescence as was obtained with PHA, was observed after incubation of thymocytes from the tumour-bearing animal with PWM (figure 4), although the response appeared to have dose dependent characteristics, reducing as the concentration of PWM increased. Thymocytes from the control animal showed a marked increase in polarization value at low

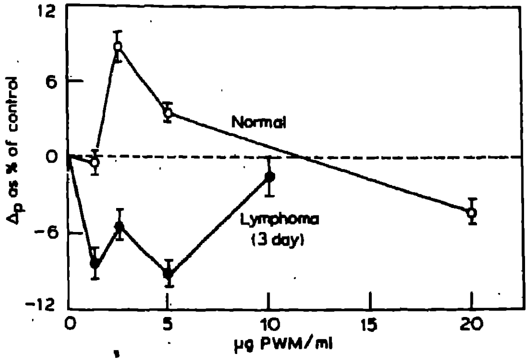

Figure 4. Change in polarization of fluorescein fluorescence for thymocytes from control and lymphoma-bearing mice, incubated with PWM (± s.e.).

concentration, reducing to a slight decrease in polarization value at higher concentrations. In addition, changes in the rate of FDA hydrolysis and leakage of fluorescein from the cells were also observed as shown in figures 5 and 6.An increase in FDA hydrolysis rate of up to 50% was observed with control thymocytes, whereas with thymocytes from the tumour bearing animal the response to PWM was the

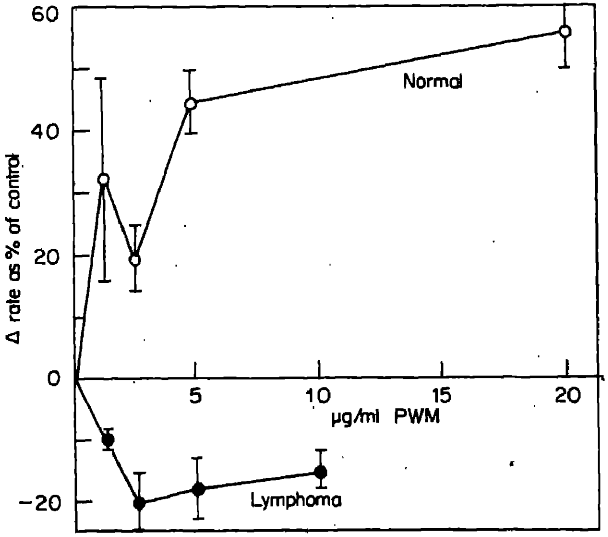

Figure 5. Effect of PWM concentration on the mean rate of FDA hydrolysis for thymocytes from normal and lymphoma bearing mice (± s.e.).

complete converse, *i.e.* a decrease in rate up to 20%. Similarly as shown in figure 6, an increase in the intracellular fluorescein concentration in normal thymocytes was observed, compared to a decrease in intracellular fluorescein level in thymocytes from the tumour bearing animal. The intracellular fluorescein concentration or *Fi*, was determined at equivalent total fluorescence levels, and therefore corresponds to a significant change in the rate of leakage of fluorescein out of the cell.

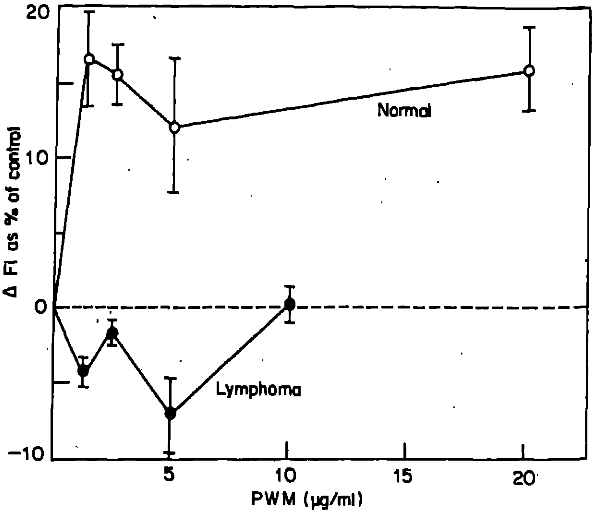

Figure 6. Change in intracellular fluorescein concentration (*Fi*) in thymocytes from normal and lymphoma-bearing mice, incubated with PWM (± s.e.).

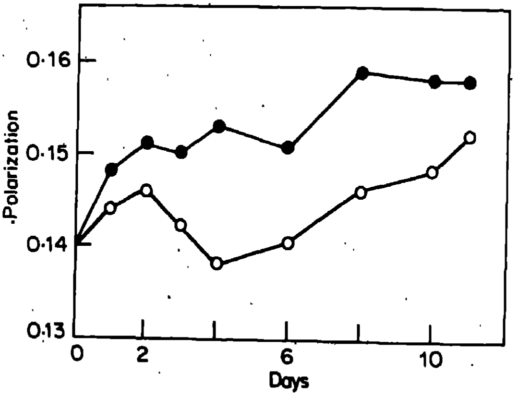

Figure 7. Change in polarization of fluorescein fluorescence in murine thymocytes during the growth of an intraperitoneal NK/LY/R lymphoma. (○), Control; (●), after incubation with PHA at a concentration of 10 μg/8× 10^6 cells/ml.

The above results were obtained using thymocytes from mice bearing a 3-day old lymphoma. Measurements were also made using thymocytes prepared from mice bearing a lymphoma for only 24 h and up to 11 days as shown in figure 7. The absolute polarization value increased from 0.14 to 0.16 as the tumour progressed, whereas the response to PGA, *i.e.* the reduction in polarization value, reached a maximum value of about 15% after about 3 to 4 days, gradually decreasing as the tumour progressed.

Discussion

Each of the cell samples studied showed a change in polarization of fluorescence with time, reaching a plateau at higher concentrations of fluorescein, whilst simultaneously the rate of accumulation of intracellular fluorescein appeared to be decreasing while the actual hydrolysis rate remained almost linear. It is tempting to ascribe this result to a concentration depolarization effect (Dale and Bauer, 1971). The distribution of fluorescein within the cell is a matter of conjecture, not accurately known, since calculations based on known (or extrapolated) fluorescein concentrations take into account only intra- and extracellular volumes and ignore interaction between fluorescein and membranes or enzymes sites. It is likely, therefore, that any calculation of fluorescein concentration will underestimate the actual local concentrations. Two hypotheses may be considered to explain the observed change in polarization value with time. One possibility is that fluorescein, as formed, is redistributed from a more rigid to a more aqueous phase thus altering the average observed polarization value. This mechanism is supported by single-cell measurements using the cytofluorograph (Udkoff and Norman, 1979). A second possibility is that fluorescein may achieve sufficiently high local concentration for quenching to be significant, with associated depolarization.

The rate of fluorescein production appears to be linar (figure 1). A saturation curve for fluorescein within the cell is also shown. The limiting value of fluorescence may arise when the rate of diffusion of fluorescein out of the cell approaches its rate of production. It is possible that FDA transport into the cell is competitively blocked by fluorescein, if both have affinity for the same structure involved in membrane transport (Steen and Lindmo, 1976). The apparent alteration in intracellular accumulation of fluorescein could be due to feedback inhibition by the reaction products. However the total fluorescence was observed to be increasing when intracellular fluorescence was still at a plateau level, suggesting that saturation or quenching may be more important than inhibition of FDA transport.

The fluorochromasia studies described here have failed to reveal a stable polarization value while fluorescence levels are increasing; indeed there is a close link between polarization values and intracellular concentrations of fluorescein which in turn are dependent upon the hydrolysis rate and the leakage of fluorescein out of the cell. The results reported here were obtained using a final FDA substrate concentration, of 2.4×10^{-6} M, with hydrolysis for up to 20 min at 27° C. Similar effects have been observed by others (Epstein *et al.*, 1971; Udkoff and Norman, 1979; Hashimoto *et al.*, 1979; Mitchell *et al.*, 1980) using similar or different measuring systems and FDA substrate concentrations.

The similarity in the response of murine spleen lymphocytes and human peripheral blood lymphocytes after PHA incubation was not unexpected, since the spleen lymphocyte population corresponds to some extent to the lymphocyte population in the mouse perhipheral system. The lack of response of murine thymocytes from normal animals to PHA or PWM and the increasing response of thymocytes from the lymphoma bearing animal seems to correspond to changes in the mature/immature cell ratio (Jenkins, 1975; Dumont, 1973) during tumour development. These changes have been illustrated by measuring the electrophoretic mobility of individual cells in the suspension as shown in figure 8. For the control animal there is a fast/slow population ratio of about 1 to 9, *i.e.* about 10% fast cells. As the tumour progresses, the ratio of fast to slow cells increases, reaching aout 1 to 2 after about 3 days. The fast population has been identified as consisting of immunocompetent thymocytes, which are released to the periphery. It is

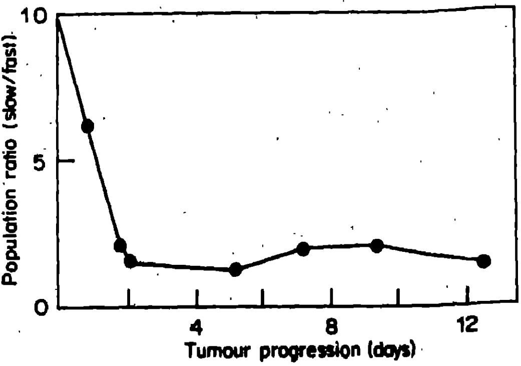

Figure 8. Change in the thymocyte population ratio (slow/fast) during the growth of an intraperitoneal NK/LY/R lymphoma, measured by cell microelectrophoresis.

possibly this population which is responding to PHA and PWM, but not to WGA. Conversely, the immature thymocyte population may show a better response to WGA and other non-mitogenic lectins.

It is therefore possible that it is the immunocompetent *T*-cell population which is responding to PHA in the spleen lymphocyte suspension and by analogy, in the human peripheral lymphocyte sample.

The thymocyte response to PHA appears to reach a maximum, long before maximum tumour load. It is therefore possible that lymphocytes from a human cancer patient may respond to PHA at one stage of the disease but not at another. Similarly, a significant population of immature lymphocytes or immunoblasts, in the peripheral circulation could lead to an abrogated response to PHA. In the early studies of many cancer detection tests, based on isolated peripheral blood lymphocytes, it is often common practice to compare the response of lymphocytes from young healthy laboratory staff with those from patients with confirmed disease, patients who are often old and in terminal care. The change in the responsiveness of murine thymocytes to PHA during the progression of the tumour lends support to the view that this is bad practice. The discrepancy between the reported results of polarization changes in lymphocytes from patients with cancer could also be related to this observation.

The mechanism of the response of lymphocytes to mitogenic lectins, as detected during fluorochromasia is still unclear. Experiments are in progress to determine whether the assay may be a quick, sensitive and non-subjective physical technique for measuring the early events of lymphocyte activation.

Acknowledgement

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Cell binding and mitogenic properties of the lima bean lectins

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Abstract. Two lectins, a tetramer designated LBL_4 and an octamer LBL_8 designated have been purified from the lima bean *Phaseolus lunatus*. The tetramer appears to be nonmitogenic for human lymphocytes and is a weak mitogen for bovine cells. The octamer and a chemically cross-linked form of the tetramer are good mitogens. The lima bean lectin binds to only certain sub-populations of human lymphocytes. The primary class which does not bind appears to be a sub-population of *T*-lymphocytes. Comparisons of cell binding with other lectins which bind to 2-acetamido-2-deoxy-D-galactose have been carried out. Quantitative analysis of the binding to human erythrocytes is co-operative but binding to lymphocytes is non-co-operative. These results show that there may not be a direct correlation between mitogenic stimulation and co-operative binding to membrane receptors.

Keywords. Lima bean; lectin; metal ions; lymphocytes; mitogenesis.

Introduction

The lectins from lima beans *Phaseolus lunatus* have been examined since 1945. Early studies showed them to be blood group specific, preferentially agglutinating type *A* human erythrocytes (Boyd and Reguera, 1949). These lectins are one of many which show specificity for binding 2-acetamido-2-deoxy-D-galactose (or N- acetyl-D-galactosamine).

The lectins have been purified by gel (Gould and Scheinberg, 1970) and affinity (Galbraith and Goldstein, 1972) chromatogrphy. This work by Gould and Scheinberg (1970) and Galbraith and Goldstein (1972) did much to establish a basis for understanding the protein structure. Two lectins are obtained, one of 240,000 daltons, and another of 120,000 daltons. These have been designated in earlier literature, after the order in which they elute from chromatographic columns, as component II and component III (Gould and Scheinberg, 1970). Each lectin is composed of 31,000-dalton monomers and we have designated the tetramer as LBL_4 and the octamer as LBL_8. These early studies identified two cysteine residues per monomer. Dodecyl sulphate electrophoresis with and without reducing agent led Gould and Scheinberg (1970) to propose that each subunit had one of its cysteines involved in a disulphide linkage to another subunit. Thus, each subunit should have one free sulphydryl. Each lectin is composed of disulphide-linked dimers;

Abbreviations used: LBL_4, Lima bean lectin tetramer; LBL_8, lima bean lectin octamer; Mops, morpholinopropanesulphonate; EDTA, ethylenediaminotetraacetate; bicine, N,N-bis(2-hydroxyethyl)-glycine.

LBL_4 is two dimers and LBL_8 is four dimers. Each dimer appears to represent the binding site for one 2-acetamido-2-deoxy-D-galactopyranoside. Although LBL_4 and LBL_8 are composed of identical subunits, no interconversion between the two forms occurs. It is, therefore, possible to work with one form and study its characteristics without having any of the other forms present. As is found with many other lectins, metal ions are required for saccharide binding activity.

LBL_8 is a potent mitogen for a variety of lymphocytes, while LBL_4 is a relatively poor mitogen in comparable systems (Reichert *et al.*, 1973; Ruddon *et al.*, 1974; Bessler *et al.*, 1976). The set of identical proteins differing apparently only in aggregation states presents two intersting probes for the mitogenic response. Both the lectin structure and the usefulness as a mitogen are two avenues of research to be pursued. In this report, we present some of our early results on studies with this lectin. For a more complete background survey on the lima bean lectins, see the comprehensive review on lectins by Goldstein and Hayes (1978).

Materials and methods

Lectins were prepared from Thorogreen lima beans (Pandolfino and Magnuson, 1980). Beans were ground in a blender and suspended in 0.1 M phosphate buffer, pH 6.8. After stirring for 1 h, the suspension was filtered and the filtrate was centrifuged. A 45–50% $(NH_4)_2SO_4$ fraction was obtained from this clarified extract. After suspension in 0.5 M NaCl, 0.01 M morpholinopropanesulphonate (Mops), pH 7.0, and dialysis against the same buffer, the sample was applied to an Ultragel AcA 34 column. LBL_4 obtained was pure as determined by gel electrophoresis. LBL_8 was almost pure by the same criteria, but sometimes required a further chromatography step using SP-Sephadex.

For metal ion binding studies the lima bean lectins were first demetallized by dialysis against 0.1 M ethylenediaminotetraacetate (EDTA), 0.5 M NaCl, pH 7.0. EDTA was removed by dialysis against 0.5 M NaCl, 0.01 M Mops, pH 7.0, which had been demetallized by passage over Chelex 100. Atomic absorption showed that Mn^{2+}, Ni^{2+}, Co^{2+}, and Zn^{2+} were negligible (<1% with respect to 31,000-dalton subunits) and Ca^{2+} was less than 4%.

Metal ion binding experiments were carried out by dialysing 1 ml of lectin against 4 ml of buffer containing ^{45}Ca or ^{54}Mn. Experiments were conducted in polypropylene tubes at 5° C with gentle agitation for 5 days.

Lima bean lectins were labelled with fluorescein by treating the lectin (3–4 mg/ml) in 0.5 M NaCl, 0.05 M N,N-bis (2-hydroxyethyl)glycine (bicine), 0.4 M 2-acetamido-2-deoxy-D-galactose, pH 8.4, with saturating concentrations of fluorescein isothiocyanate. Following treatment for 16 h at 4° C, dialysis against 0.5 M NaCl, 0.01 M Mops, pH 7.0, gave labelled lectin with essentially 100% of the original agglutination activity. Other fluorescein-labelled lectins were purchased from Sigma Chemical Co., St. Louis, Missouri, USA.

LBL_4 was cross-linked with dimethyl suberimidate. To 1 ml of LBL_4, 4–10 mg/ml in 0.5 M NaCl, 0.2 M triethanolamine, 0.4 M 2-acetamido-2-deoxy-D-galactose, pH 8.5, was added 5 mmol aliquots of suberimidate. After 5 additions during a 12-h period, the reaction mixture was maintained at 4° C for another 24 h and then dialysed against 0.5 M NaCl, 0.01 M Mops, pH 7.0.

Iodination of the lectins was done following protection of the free sulphydryls with mercuric ion. Lectin (4–10 mg/ml) in 0.3 M NaCl, 0.05 M bicine, pH 8.2, was treated with a slight excess of $HgCl_2$ (with respect to 31,000 subunits). Lectin solution was then added to a glass scintillation vial with 150 μg of Iodogen (Pierce Chemical) coating the inside. To the solution was added 0.5–2 mCi of $^{125}I^-$ and after 10 min at 0° C, a two-fold excess of dithiothreitol was added. This solution was extensively dialysed first against 0.05 M NaCl, 0.01 M Mops, 10^{-5} M dithiothreitol, pH 7.0, and then against phosphate-buffered saline, pH 7.2. Lectin thus treated retained 100% of its haemagglutination activity.

Human peripheral blood lymphocytes were prepared by Ficoll-Hypaque density centrifugation (Boyum, 1968). Viability of cells was always greater than 95% as determined by trypan blue exclusion. Lymphocyte fractions enriched for *T* or *B* cells were obtained by E-rosette separation (Brown *et al.*, 1976). E-rosettes were subjected to three centrifugations through Ficoll-Hypaque, and residual monocytes were depleted by culturing cells overnight in glass Petri dishes. Monocyte identification was carried out with fluoresceinated dextran (Berlin and Oliver, 1980). Rhodamine-labelled rabbit $(F_{ab}')_2$ fragments directed against human IgM and used here to identify *B* cells were a gift from Dr. Janet Oliver of the University of Connecticut Health Centre, Farmington, Connecticut, USA. Lymphocytes were cultured in RPMI 1640 supplemented with 100 units/ml penicillin, 100 μg/ml dihydrostreptomycin sulphate, and heat-inactivated foetal calf serum. Cells were maintained at 37° C under 5% CO_2 in microtiter plates. After 72 h, 1 μCi of $[^3H]$-thymidine (6.67 Ci/mmol) was added and cells were harvested 24 h later. In some cases, when noted, cells were cultured in Iscove-Melcher media (Iscove and Melcher, 1978). Bovine lymph node lymphocytes were obtained from lymph nodes of freshly slaughtered cattle by passage over glass wool (Peters, 1975). Cells were cultured in RPMI 1640 or Iscove-Melchers media. Human erythrocytes were obtained from normal healthy volunteers. Cells were washed in phosphate-buffered saline with 10 mg/ml human serum albumin.

For lectin binding studies, cells were suspended in phosphate-buffered saline with 10 mg/ml bovine serum albumin and 1 mg/ml glucose. ^{125}I-labelled lectin was added to lymphocytes ($5–18 \times 10^7$/ml) or erythrocytes ($8–20 \times 10^8$/ml) in plastic culture tubes and after 90 min, 330 μl of cell suspension was placed above 60 μl of Dow Corning 704 silicone oil in a 40 μl microfuge tube. Tubes were centrifuged in a Beckman Microfuge B, frozen in dry ice/acetone, and cut at the oil-water interface. Cells were pelleted below the interface, and free and bound lectin were determined directly by counting water and oil phase, respectively. Experiments to determine nonspecific binding were carried out in the presence of 0.2 M 2-acetamido-2-deoxy-D-galactose or used lectin inactivated with N-ethyl maleimide. Nonspecific binding, always less than 10% of total, has been subtracted in all experiments reported.

Results

Characteristic of the lima bean lectins

The lectins LBL_4 and LBL_8 are similar to those described earlier by Gould and Scheinberg (1970) and Galbraith and Goldstein (1972). Gel electrophoresis under non-denaturing conditions gave a single, but different, band for each of the two lectins. Electrophoresis with dodecyl sulphate and 2-mercaptoethanol gave one band apparently identical for each of the lectins. The lectins appear to be identical in Ouchterlony diffusion tests with antibodies raised to each lectin independently (Galbraith and Goldstein, 1972; Pandolfino and Magnuson, 1980) and CD spectra of both are identical (Pandolfino and Magnuson, 1980). The two lectins, although composed of identical subunits, are not interconvertible. Homogeneous LBL_4 and homogeneous LBL_8 were subjected to various concentrations of urea and reducing agent. In analysis by non-denaturing electrophoresis at pH 4.3, after extensive dialysis against pH 7.0 buffer, no LBL_4 was found in LBL_8 solutions and *vice versa*.

Tow-dimensional gel electrophoretic analysis (O'Farrell, 1975) has been carried out on LBL_4 and LBL_8. All analysis gave identical patterns for LBL_4 and LBL_8. When the original system of O'Farrell (1975) was employed, no heterogeneity was observed for molecular weight but marked heterogeneity was observed for iso-electric points of the 31,000-dalton monomers. We also carried out the 2-D- analysis and omitted 2-mercaptoethanol and dithiothreitol from all the reagents, from initial extracts of the bean to final electrophoresis steps. We expected to observe less heterogeneity as disulphide-linked dimers should have migrated as units in the first dimension and spots with molecular weights approximately corresponding to dimers should have been observed in the second dimension. This result would have been expected if all monomers were linked to second monomers through a disulphide bond. A large amount of monomer was detected even when the lectin had not been exposed to any reducing agent.

Metal ion binding studies

Like many lectins, LBL_4 and LBL_8 require metal ions for saccharide binding activity. We chose to examine the stoichiometry of Mn^{2+} and Ca^{2+} binding to the lectins. Detailed Scatchard analysis were done on LBL_4 but only saturating stoichiometry was determined for LBL_8. This was done because of relatively low amount of LBL_8 available. Figure 1 shows the results for Mn^{2+} binding to demetallized LBL_4, Ca^{2+} binding to demetallized LBL_4 and Ca^{2+} binding to LBL_4 presaturated with Mn^{2+}. This binding study shows that there are 4 metal binding sites per tetramer of LBL_4. Two sites can be occupied by Mn^{2+} and the remaining two with Ca^{2+}. Alternately, all 4 sites can be filled with Ca^{2+}. When only Ca^{2+} is binding to the lectin, binding is positively co-operative, but co-operativity is not observed with either Mn^{2+} binding or Ca^{2+} binding to LBL_4 with Mn^{2+} already present. This interpretation has assumed that all sites might bind either Ca^{2+} or Mn^{2+}. This is the simplest interpretation but other possibilties do exist.

Mitogenic characteristics of LBL_4 and LBL_8

Mitogenic stimulation of lymphocytes by the lima bean lectins had been demonstrated early (Reichert *et al.*, 1973) and more recent reports have examined the characteristics in more detail (Ruddon *et al*, 1974; Bessler *et al.*, 1976). We have examined the stimulation of human lymphocytes by LBL_4, LBL_8, and the cross-linked LBL_4. This product of suberimidate cross-linking was actually a mixture of octamers and higher aggregates. Unreacted LBL_4 was separated by chromatography on Ultragel AcA 34. For human lymphocytes, LBL_4 was found not to be mitogenic, LBL_8 was a good mitogen, and the cross-linked LBL_4 closely resembled LBL_8. Mitogen stimulation increased with increasing levels of lectin.

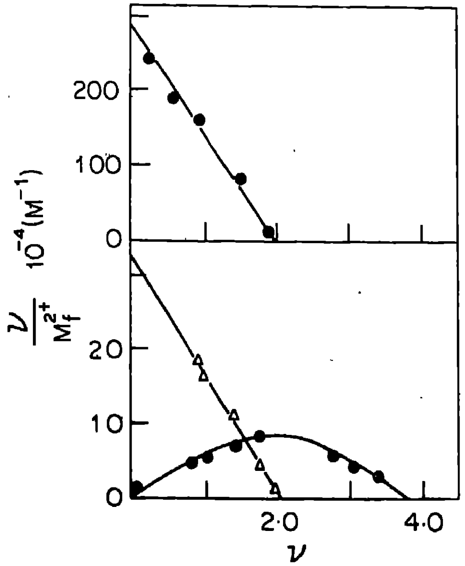

Figure 1. Scatchard plots of Mn^{2+} and Ca^{2+} binding to demetallized LBL_4. Top panel: Binding of Ca^{2+} to demetallized LBL_4 pretreated with Mn^{2+}. Panel B: Binding of Mn^{2+} to demetallized LBL_4 (Δ); binding of Ca^{2+} to demetallized LBL_4 (●). Ratio of bound metal ions to total LBL_4 molecules is represented by v. M_f^{2+} is free molar concentration of either Ca^{2+} or Mn^{2+}.

For cells cultured at 2×10^6/ml, maximum stimulation was reached at concentrations of 25 μg/ml with a constant level of stimulation being observed at 25, 50, and 100 μg/ml.

Fractionation of human peripheral blood lymphocytes by E-rosetting was used to give enriched populations of *T*- and *B*-lymphocytes. The *T*-lymphocyte response to LBL_8, was high, but the *B*-lymphocyte response was reduced. In addition, blasts from stimulated cultures were shown to form rosettes with sheep red blood cells showing that the blasts were stimulated *T* cells (Abauf and Daguillard, 1977). When monocytes were depleted from the E-rosette fraction by adherence to glass dishes, the mitogenic response was reduced to background but could be restored by supplementation with unfractionated cells.

A lectin with similar specificity to the lima bean lectins is soybean agglutinin. Previous studies had shown that soybean agglutinin was not mitogenic to untreated human lymphocytes but would become mitogenic if cells were treated with neuraminidase. We compared mitogenic stimulation of LBL_4 and LBL_8 with untreated and neuraminidase-treated cells. These neuraminidase-treated cells were stimulated by soybean agglutinin. No mitogenic activity was observed for LBL_4 and the activity for LBL_8 was unchanged with these treated cells.

Our work with bovine lymphocytes is not as extensive as with human cells. A dose response curve for bovine lymph node lymphocytes is shown in figure 2. LBL_8 is a good mitogen while LBL_4 is a weak mitogen.

Cell binding characteristics of the lima bean lectins

Binding to cell surfaces was first investigated by using LBL_4 and LBL_8 which had been labelled with fluorescein. Competition experiments were set up in which

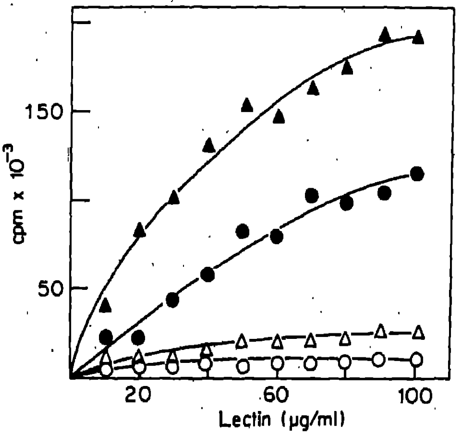

Figure 2. Mitogenic response for bovine lymph node lymphocytes stimulated with various doses of LBL. LBL_4 at various concentrations in RPMI 1640 supplemented with 50% foetal bovine serum. ○;and LBL_4 in Iscove-Melchers media, Δ. LBL_8 at various concentrations in RPMI 1640 supplemented with 5% foetal bovine serum, (●); and LBL_8 in Iscove-Melchers media, (▲).

Table 1. Binding of fluorescein-labelled lectins to lymphocytes.

Treatment[a]	Cells labelled %
Fl-LBL_4	66 ± 5
Fl-LBL_8	66 ± 12
Fl-SBA	1
Fl-L-PHA	99
Fl-LBL_4 *vs.* LBL_8	1
Fl-LBL_8 *vs.* LBL_4	1
Fl-LBL_4 *vs.* SBA	66 ± 1
Fl-LBL_8 *vs.* L-PHA	1
Fl-L-PHA *vs.* LBL_8	99

[a] In competition experiments, human lymphocytes were treated with unlabelled lectin (1.5–20 mg/ml) for 15 min at 0°C prior to addition of fluorescein-labelled lectin.

human lymphocytes were pretreated with an unlabelled lectin and then treated with a labelled lectin. Results of these experiments are shown in table 1. LBL_4 and LBL_8 only bind to approximately 70% of all human lymphocytes. They clearly bind to the same receptor as pretreatment of the cells with non-fluorescent lectin prevents binding of fluorescent lectin in subsequent treatments. Soybean agglutinin binds almost to no cells and also has no effect on subsequent binding of fluorescent LBL_8. The lectin PHA binds almost to all cells and prevents subsequent binding of fluorescent LBL_8. LBL_8, however, does not block binding of fluorescent PHA. Of course, these fluorescence experiments are not quantitative in the sense that they do not easily detect reduced levels of binding.

Neuraminidase treatment of the cells did alter some of the binding patterns. Although the mitogenic response to LBL_4 was not altered, as indicated earlier, essentially 100% of the lymphocytes bound fluorescent LBL_4 and LBL_8 after treatment. Before neuraminidase treatment, we could detect no binding by fluorescent soybean agglutinin, but after treatment essentially 100% of all lymphocytes bound the fluorescent lectin. About 40% of cells binding soybean agglutinin would not bind this agglutinin after treatment with LBL_8.

We attempted to compare the quantitative aspects of LBL_4 and LBL_8 binding to lymphocytes to establish, whether or not, the mitogenic capability of the two was reflected in binding characteristics. Bovine lymph node lymphocytes were used because large quantities of cells were needed. As shown in figure 2, LBL_8 is a potent mitogen and LBL_4 is only a weak mitogen for bovine cells. Binding analysis were done using iodinated LBL_4 and LBL_8. The results of binding assays are shown in figure 3. The binding is characterized by one type of site for both LBL_4 and LBL_8. Affinities are approximately the same for each. LBL_4 has an association constant of 4×10^5 M^{-1}, and LBL_8 has a constant of 6×10^5 M^{-1}. Approximately 50% more LBL_4 molecules can bind to the cell surface but a larger mass of LBL_8 can bind.

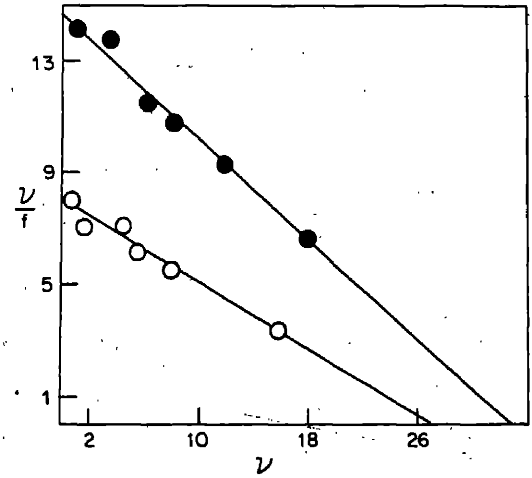

Figure 3. Representative plot for binding of LBL_4 (○) and LBL_8 (●) to bovine lymph node lymphocytes obtained from a single animal. The value of is the ratio of 60,000-dalton dimers/cell where dimer represents one saccharide binding site. Values presented are actual values multiplied by 10^{-5}. The value of *f* is molar concentration of dimers. The /*f* values presented have been multiplied by 10^{-11}.

Discussion

We have described a relatively easy method for the purification of lima bean lectins to homogeneity as shown by gel electrophoresis. Mitogenic characteristics, molecular size, and sugar binding specificity are similar to those reported by earlier workers. LBL_4 and LBL_8 appear to be made of identical protomers and vary only with respect to degree of aggregation. We have subjected the lectins to two-dimensional gel electrophoresis analysis and find that a simple model in which all monomers are cross-linked through disulphides into dimers may not hold for the lectin we have purified. We found significant amount of monomers in our preparations. Variations in lectin structure may exist when comparing different strains of lima beans. We have found that agglutination activity in extracts varies significantly with the strain of the bean. In studies using antisera to the lima bean lectin we have also observed grossly varying amounts of lectin in antibody-antigen precipitin tests. Variation with source of the lectin is not entirely surprising. The lentil lectin is a good example of previously demonstrated variations (Goldstein and Hayes, 1978).

Early experiments by Bessler and Goldstein (1974) demonstrated that each LBL_4 molecule has two saccharide binding sites and each LBL_8 had four sites. Our metal ion binding studies indicate also that stoichiometry associated with these lectins is reflected in half the number of subunits present. We find that when Mn^{2+} is present, only two Mn^{2+} and two Ca^{2+} ions bind to each LBL_4 tetramer. Although Ca^{2+} can replace the Mn^{2+}, strong interaction between the subunits is then observed.

The pairing of LBL_4 with LBL_8 poses interesting possibilities for studying cell binding and mitogenic characteristics. Our fluorescent lectin studies show that LBL_4 and LBL_8 bind to the same cell receptor. Receptor binding, in itself, is not enough to initiate the mitogenic response as LBL_4 is a weak mitogen. The work on cross-linked LBL_4 supports the concept that receptor cross-linking is very important for mitogenic stimulation of lymphcoytes. The competition studies with other lectins again indicate that cell receptor binding cannot be totally explained by specificity to a single monosaccharide.

Prujansky *et al.* (1978) have proposed a model for mitogenic stimulation which requires that binding of a mitogenic lectin to a lymphocyte surface should demonstrate positive co-operativity. We felt that comparisons of LBL_4, a very weak mitogen, and LBL_8, a potent mitogen, would be valuable in testing the above hypothesis. Interestingly we found that neither LBL_4 nor LBL_8 bound co-operatively to bovine lymphocytes, although it was easily demonstrated that binding to human type A erythryocytes is positively co-operative (unpublished observations). Based on our results, we believe that co-operativity in binding may not be an absolute requirement for mitogenesis.

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Structural basis for the affinity of four insolubilized lectins, with a specificity for α-D-mannose, towards various glycopeptides with the N-glycosylamine linkage and related oligosaccharides

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Abstract. Precisions are given on the fine specificity and on the usefulness of immobilized concanavalin A, *Lens culinaris*, *Vicia faba* and *Pisum sativum* agglutinins for fractionation of glycopeptides with the N-glycosylamine linkage.

While insolubilized concanavalin A represents a very useful tool for the fractionation of both N-acetyllactosaminic and oligomannosidic type glycopeptides or related oligosaccharides, immobilized *Lens culinaris*, as well as *Vicia faba* or *Pisum sativum* agglutinins allow the subfractionation of some N-acetyllactosaminic glycopeptide populations on the basis of the presence of an α-L-fucose residue substituting in C-6 position the N-acetylglucosamine residue involved in the N-glycosylamine bond.

Keywords. Affinity chromatography; specificity of lectins; fractionation of glycopeptides.

Introduction

Lectins are now widely used as tools to visualize the topography of saccharide moieties on cell surfaces (Molday, 1981), or to follow by lectin-affinity immunoelectrophoresis variations in the glycosylation of plasma (Bøg-Hansen *et al.*, 1975) or membrane glycoproteins (Bjerrum and Bøg-Hansen, 1976). Insolubilized on various supports, lectins are also widely used to fractionate soluble or membrane glycoproteins of diverse origins (Lotan and Nicolson, 1979) as well as glycopeptides obtained from soluble or membrane-bound glycoproteins (Finne *et al.*, 1980).

Among the most used lectins are the so-called α-D-mannose or glucose binding lectins: Concanavalin A (Con A), *Lens culinaris* agglutinin (LCA), *Vicia faba* agglutinin (VFA) and *Pisum sativum* agglutinin (PSA) (Goldstein and Hayes, 1978). However, it is now well known that it is not only a monosaccharide residue in a terminal non reducing position on a glycan that is recognized by a given lectin, but more complex saccharidic sequences (Kornfeld and Ferris, 1975).

Abbreviations used: Con A, Concanavalin A; LCA, *Lens culinaris* agglutinin; VFA, *Vicia faba* agglutinin; PSA, *Pisum sativum* agglutinin; GP-h-STF, glycopeptide isolated from human serotransferrin; GP-h-LTF, from human lactotransferrin; GP-ovoTF, hen ovotransferrin; GP-b-LTF, bovine lacto transferrin; FNR, non retained profile, FR, retained profile; FE, sharp elution profile; FTB, "tightly bound" profile.

In previous studies (Debray *et al.*, 1979, 1981), we used oligosaccharides or glycopeptides related to glycoproteins with the N-glycosylamine linkage (Montreuil, 1980, 1982) to precise the specificities of various lectins and in particular of the above α- D-mannose binding lectins, by inhibition of agglutination of human red blood cells. These results were completed by studying the affinity of the insolubilized lectins towards these oligosaccharides or glycopeptides.

Materials and methods

Lectins

All the lectins used in this study, except concanavalin A (Con A) which was purchased from Pharmindustrie, Clichy, France, have been purified by affinity chromatography.

Origin of glycopeptides and oligosaccharides

Oligosaccharides (N-9, N-5, N-6, S-6a) shown in figure 1 were isolated by Dr G. Strecker from the urines of patients with various lysosomal diseases (Strecker and Montreuil, 1979). Glycopeptides isolated from human serotransferrin (GP-h-STF), from human lactotransferrin (GP-h-LTF$_1$) and from hen ovotransferrin (GP-ovoTF) were a gift from Prof. G. Spik. The glycopeptide of the oligomannosidic type, isolated from bovine lactotransferrin (GP-b-LTF) was a gift from Dr A. Chéron.

All these structures shown in figure 1 are related to glycoproteins with the N-glycosidic linkage and belong to 2 types of glycan structures: the oligomannosidic and the N-acetyllactosaminic type.

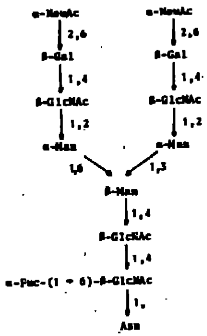
In the first family, the common pentasaccharidic core is substituted uniquely by mannose residues to give the oligomannosidic type as found in bovine lactotransferrin (GP-b-LTF of figure 1) or by a variable number of residues of N-acetyllactosamine and of sialic acid and/or fucose to give the N-acetyllactosaminic type (Montreuil, 1980, 1982). In the case of human serotransferrin, we found two of these N-acetyllactosamine sequences substituted by two residues of sialic acid, giving a bi-antennary N-acetyllactosaminic type glycan (GP-h-STF of figure 1).

With one or two other N-acetyllactosaminic sequences, are obtained the tri- or tetra-antennary glycans as those characterized in human α_1-acid glycoprotein (Fournet *et al.*, 1978). Very often, on a bi-antennary glycan, a L-fucose residue is branched in C-6 position on the N-acetylglucosamine residue involved in the N-glycosylamine bond. This type of glycan has been found in human lactotransferrin (GP-h-LTF of figure 1) (Spik *et al.*, 1982). Sometimes, the core β-mannose residue is substituted by an intersecting β-N-acetylglycosamine residue as in the glycan isolated from hen ovotransferrin (GP-ovoTF of figure 1).

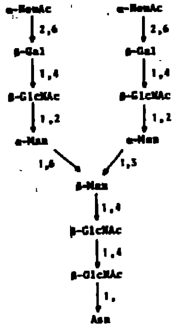
Haemagglutination tests

Agglutination of human red blood cells group O$^+$ and haemagglutination inhibition by various oligosaccharides or glycopeptides were carried out according to Matsumoto and Osawa (1970). Results were expressed as the minimum concentration of sugar (mM) necessary to completely inhibit agglutination.

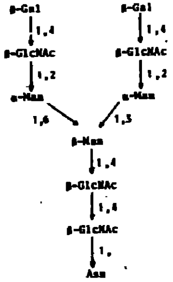
GP-hLTF-1



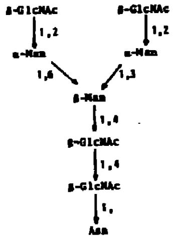
GP-hSTF



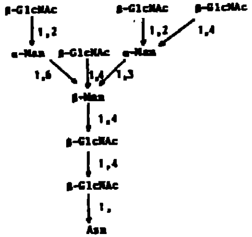
Asialo-GP-STF



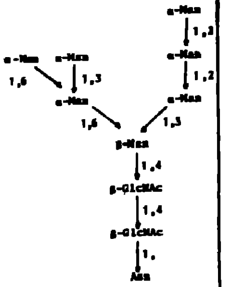
Asialo-agalacto-GP-STF



GP-ovo IV

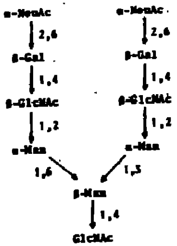


GP-hLTF

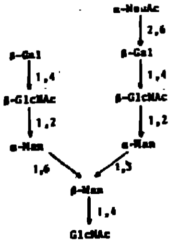


	GP-hLTF-1	GP-hSTF	Asialo-GP-STF	Asialo-agalacto-GP-STF	GP-ovo IV	GP-hLTF
Con A	0.103	0.103	0.103	0.062	0.207	0.0062
LCA	0.0015	0.0125	0.0125	> 3.33	1.66	0.207
VFA	0.0031	0.0515	0.0515	> 3.33	0.207	0.83
PSA	0.0031	0.0515	0.0515	> 3.33	N.D.	0.415

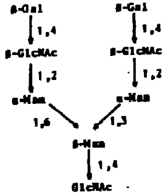
N-9



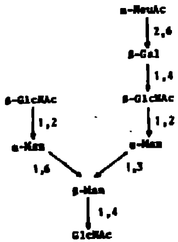
N-5



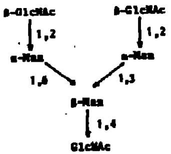
Asialo-N-9



N-6



S-6a



	N-9	N-5	Asialo-N-9	N-6	S-6a
Con A	0.103	0.103	0.0515	0.025	0.0012
LCA	1.66	0.35	0.415	0.103	0.63
		0.175	0.207	0.103	1.24

Insolubilization of lectins on Sepharose 4B

Lectins were coupled to Sepharose 4B activated according to the procedure of March *et al.* (1974), at a concentration of 2 mg of lectin per ml of gel.

Affinity chromatography of oligosaccharides or glycopeptides on lectin-Sepharose column

Glycopeptides, N-^{14}C acetylated according to Koide *et al.* (1974), or related oligosaccharides, reduced on the terminal N-acetylglucosamine residue with tritiated sodium borohydride (Takasaki and Kobata, 1974) were applied, after dissolution in phosphate buffered saline pH 7.4 (LCA, VFA, PSA) or in 0.005 M sodium acetate buffer pH 5.2 containing 0.1 M·NaCl and $CaCl_2$, $MgCl_2$ (1 mM each) in the case of insolubilized Con A, to the column of lectin-Sepharose (10×1 cm) equilibrated with the same buffer. Elution was carried out first with buffer and then with buffer containing 0.15 M methyl-α-D-glucoside. Fractions of 2 ml were collected and samples of 100 μl analysed for radioactivity.

Results and discussion

Haemagglutination inhibition results

These results are summarized in figure 1. The best inhibitors found for Con A are asialo-agalacto GP-h-STF and the related oligosaccharide S-6a and Con A presents a great affinity for the trimannosidic core substituted by two N-acetylglucosaminyl residues. The affinity of the lectin decreases when these N-acetylglucosamine residues are substituted by one or two galactose residues as in asialo-GP-STF and asialo N-9 (figure 1) and by one or two sialic acid residues as in GP-h-STF and N-9 of figure 1. It is noteworthy that this affinity, as measured by inhibition of agglutination, is not decreased by the addition of a β-1,4-linked N-acetylglucosamine on the β-linked mannose residue. On the other hand, Con A also presents a great affinity for GP-b-LTF which possesses the complete trimannosidic core with α-1,3 and α-1,6-mannose substituted by four additional α-linked mannose residues (oligomannosidic structure). Substitution of core β-1,3-mannose at position C-4, as in GP-ovoTF, decreases the affinity of Con A.

However, the best inhibitor found for the three other lectins LCA, VFA and PSA is glycopeptide GP-h-LTF$_1$ and the α-1,6-linked fucose residue seems to be a major determinant of the binding, for its removal with an α-L-fucosidase from rat kidney gives glycopeptides 10-fold less inhibitory (GP-h-STF). However, if presence of an α-1,6-linked fucose near the N-glycosidic linkage seems to be a necessary condition, its presence is not a sufficient one, and glycopeptides possessing this fucose residue, but where terminal neuraminyllactosamine sequences are lacking are very poor inhibitors (Debray *et al.*, 1981). This suggests the existence of an extended carbohydrate binding site for LCA, VFA and PSA and the methyl group of L-fucose near the N-glycosidic linkage could interact with an hydrophobic area in or near this carbohydrate binding site. This hydrophobic area had been previously suggested for LCA, VFA and PSA by Allen *et al.* (1976). Such an hydrophobic region, close to the carbohydrate-binding site had also been demonstrated for Con A (Loontiens *et al.*, 1973).

Affinity chromatography of glycopeptides with the N-glycosylamine linkage and related oligosaccharides on insolubilized lectins

The haemagglutination inhibition values given in figure 1 can be related to the true association constants between various oligosaccharides or glycopeptides and a given lectin, and from these inhibition values the affinity of a saccharide for the insolubilized lectin could be predicted.

In fact, for insolubilized Con A, results obtained by this second method, confirms previous data (Ogata *et al.*, 1975; Baenziger and Fiete, 1979). The four elution profiles which can be obtained with insolubilized Con A are shown in figure 2: i) The non-retained profile (FNR) is obtained with tetra- or tri-antennary glycopeptides of the N-acetyllactosaminic type or with bi-antennary structures possessing an additional β-1,4-linked N-acetylglycosamine on the β-linked mannose residue. One can note that this last point is in contrast with the haemagglutination inhibition results, where this β-1,4 substituted glycan possesses the same inhibitory power than the unsubstituted one (S-6a). This discrepancy is not explained.

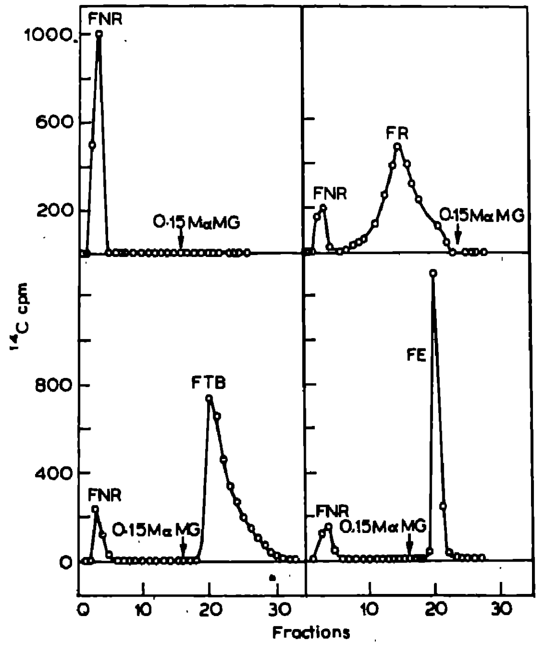


Figure 2. Con A—Sepharose elution profiles.

ii) The retarded profile (FR) is obtained by elution with the starting buffer and reflects a weak affinity between Con A and the saccharide. This profile is obtained with bi-antennary structures as GP-h-STF (Debray and Montreuil, 1978; Narasimhan *et al.*, 1979). However, with a higher concentration of Con A in the gel, these structures can be weakly retained and then eluted specifically with low concentration of α-methyl-D-glucoside or mannoside (Krusius *et al.*, 1976).

iii) The sharp elution profile (FE) with 0.3 M α-methyl-D-glucoside is obtained with oligomannosidic type glycans as GP-h-LTF (Debray and Montreuil, 1978;

Narasimhan *et al.*, 1979). However, with a higher concentration of Con A in the gel, a broad peak can be obtained (Krusius *et al.*, 1976) and some glycans cannot be eluted with α-methyl-D-mannoside but only with detergents (Ogata *et al.*, 1975).

iv) The "tightly bound" profile (FTB) is obtained with bi-antennary structures such as S-6a or asialo-agalacto-GP-h-STF, where at least one N-acetylglucosamine β (1 → 2) α-mannose sequence is very accessible to the lectin. Same results were described by Narasimhan *et al.* (1979) and Baenziger and Fiete (1979) and this broad, trailing profile expresses a very great affinity between the saccharide and the lectin.

With insolubilized LCA, VFA or PSA, three elution profiles shown in figure 3 can be obtained:

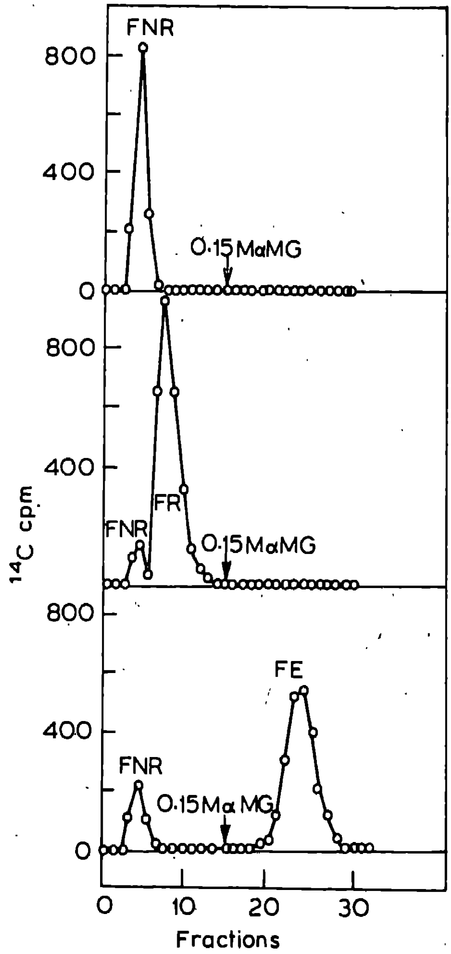


Figure 3. LCA, VFA or PSA — Sepharose elution profiles.

(FNR, Non-retained profile; FR, retained profile; FTB, tightly bound profile; FE, sharp elution profile; α-MG, α-methyl glucoside).

i) The non-retained profile (FNR) is obtained with tetra-, tri- or bi-antennary glycopeptides lacking the α-1,6-linked fucose on the N-acetylglucosamine involved in the N-glycosylamine bond.

ii) The retarded profile is obtained for insolublilized VFA or PSA, with bi-antennary glycopeptides (GP-h-LTF) possessing this α-1,6-linked fucose:

iii) This glycopeptide (GP-h-LTF) is tightly bound (FTB) on insolubilized LCA and is only displaced with 0.15 M α-methyl-N-glucoside.

In the case of insolubilized LCA, after desialylation of GP-h-LTF, the affinity of the lectin for the new structure is not modified. But, after removal of the two galactose residues with a β-galactosidase, the affinity of LCA increases and a broad trailing profile is obtained, reflecting a very great affinity between the saccharide and the lectin. Then, after removal of the N-acetylglucosamine residues with a β-N-acetylglucosaminidase, the affinity of the lectin decreases and finally, if one of the two α-mannose residues is taken off with an α-mannosidase, the remaining structure is no longer retained on the insolubilized LCA.

So, in fact, the saccharidic sequence recognized by LCA and also by VFA and PSA is the pentasaccharidic core substituted by two β-1,2 N-acetylglucosamine residues and possessing an α-L-fucose residue in C-6 position on the N-acetylglucosamine residue involved in the N-glycosylamine bond. However, if the presence of this α-1,6-linked fucose seems to be a necessary condition for the recognition by LCA or PSA and VFA, its presence is not a sufficient one, for the glycan, liberated from glycopeptide GP-h-LTF by hydrazinolysis and which still posssess this α-1,6-linked fucose, is no longer retarded on VFA and PSA or retained on insolubilized LCA. This shows the importance of the N-acetyl glucosamine-asparagine sequence in the binding reaction and this can be related to the fact that the chitobiose core gives to the whole glycan a more rigid conformation as opposed to the random configuration of the oligosaccharide obtained by hydrazinolysis.

These interactions between glycopeptides such as GP-h-LTF and LCA can also involve charge effects and (or) some hydrophobic interactions between an hydrophobic area in or near the carbohydrate binding site of the lectin and aromatic residues of the peptidic part of the glycopeptides or glycoproteins.

These results on the specificities of LCA and PSA were recently confirmed and extended by Kornfeld *et al.* (1981), who have shown that LCA or PSA can interact also with tri-antennary glycopeptides of the N-acetyllactosaminic type, possessing an α-L-fucose residue substituting in C-6 position the N-acetylglucosamine residue involved in the N-glycosylamine bond, but an important condition for this interaction to take place is that this third branch substitutes a core α-mannose residue in the C-6 position. If the substitution is in C-4 position, LCA or PSA do not show any affinity for these new tri-antennary structures.

Conclusion

Our results show that four lectins (Con A, LCA, VFA and PSA), considered to be identical in terms of α-D-mannose or glucose specificity, possess the ability to recognize fine differences in more complex carbohydrate structures. While insolubilized Con A represents a very useful tool for the fractionation of both N-acetyllactosaminic and oligomannosidic type glycopeptides or related oligosaccharides, the three others lectins will also be very useful for the separation of N-acetyllactosaminic type glycopeptides on the condition that an α-L-fucose residue substitutes in C-6 position the N-acetylglucosamine residue involved in the N- glycosylamine bond.

Acknowledgements

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Studies of the synthesis, structure and function of the phosphorylated oligosaccharides of lysosomal enzymes

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Abstract. Newly synthesized lysosomal enzymes were found to contain N-acetylglucosamine residues in phosphodiester linkage to the 6 position of the mannose residues on high-mannose type oligosaccharides. The formation of these structures was shown to be catalyzed by a specific N-acetylglucosaminylphosphotransferase enzyme, that utilises UDP-N-acetylglucosamine as a donor. The phosphorylation reaction can take place on any of four or five positions on the high-mannose oligosaccharide. Subsequently an α-N-acetylglucosaminylphosphodiesterase removes the outer blocking N-acetylglucosamine residues to generate the mature phosphomannsoyl recognition signal. This signal is responsible for the targetting of newly synthesized lysosomal enzymes to lysosomes. The human syndromes of I-cell disease (Mucolipidosis II) and pseudo-Hurler polydystrophy (Mucolipidosis III) were shown to be caused by deficiency of the first enzyme in the pathway, the UDP-N-acetylglucosamine: Glycoprotein N-acetylglucosaminylphosphotransferase.

Keywords. Lysosomal enzymes; I-cell disease; phosphorylated oligosaccharides; glycoproteins.

Previous studies have shown that a common phosphomannosyl recognition marker present on lysosomal enzymes appears to be important in their endocytotic uptake and their intracellular targetting (Kaplan *et al.*, 1977; Sando and Neufeld, 1977; Kaplan *et al.*, 1978; Natowicz *et al.*, 1979; Distler *et al.*, 1979; von Figura and Klein, 1979; Bach *et al.*, 1979; Fischer *et al.*, 1980; Hasilik and Neufeld, 1980). In order to examine the structure of this recognition marker, we labelled mouse lymphoma cells for 3 h with [2-^{3}H]-mannose, and then immunoprecipitated the lysosomal enzyme β-glucuronidase. The high mannose oligosaccharides of the enzyme were released by endo-β-N-acetylglucosaminidase H and then fractionated into neutral and negatively charged species on QAE-Sephadex. The negatively charged oligosaccharides from newly synthesized β-glucuronidase were found to contain phosphate residues in diester linkage, with outer 'blocking' α-N-acetylglucosamine residues (Tabas and Kornfeld, 1980). Similar findings have been reported by Hasilik *et al.* (1980).

We next performed detailed structural studies on similar oligosaccharides isolated from whole-cell labelled material. These studies revealed a whole family of such phosphorylated oligosaccharides, all of which had a high-mannose type oligosaccharide core. Individual molecules contained one or two phosphate residues, either as phosphomonoesters, or as phosphodiesters, with outer 'blocking' α-N-acetylglucosamine residues. The phosphate residues were present at five possible positions on the high mannose oligosaccharide, thus generating many different isomers. The phosphomonoester containing molecules had fewer mannose residues, suggesting that they were the "oldest", and had undergone oligosaccharide processing (Varki and Kornfeld, 1981).

These findings, along with other previous evidence suggesting that the mature phosphomannosyl recognition marker contained alkaline phosphatase-susceptible phosphomonoesters (Kaplan *et al.*, 1977; Sando and Neufeld, 1977; Kaplan *et al.*, 1978; Natowicz *et al.*, 1979; Distler *et al.*, 1979; von Figura and Klein, 1979), suggested that removal of the outer N-acetylglucosamine residues must occur *in vivo*. We therefore searched for and identified a rat liver α-N-acetylglucosaminylphosphodiesterase that catalyzes such a reaction. We have now purified this enzyme about 1800-fold from rat liver, using subcellular fractionation, differential detergent extraction, DEAE-cellulose and heparin-Sepharose chromatography, concanavalin A-Sepharose affinity chromatography, and gel filtration on Sephacryl S-300. The purified preparation is completely free of a previously described lysosomal α-N-acetylglycosaminidase (Weismann *et al.*, 1967). The enzyme is a smooth membrane bound glycoprotein that migrated as a single form in all the purification steps. It has a broad pH optimum, between 6.0–8.0, and is unaffected by divalent cations or reducing agents. It is capable of removing α-linked N-acetylglucosamine residues from all of the five positions on the high mannose oligosaccharides that we had previously found. It is also capable of removing α-N-acetylglucosamine residues from phosphodiester linkage in molecules such as UDP-N-acetylglucosamine, but had no activity against *p*-nitrophenyl-α-N-acetylglucosaminide (Varki and Kornfeld, 1980, 1981).

We have also identified the UDP-GlcNAc : glycoprotein N-acetylglucosamine-1-phosphotransferase that carries out the phosphorylation reaction. The basis of the assay was to follow incorporation of ^{3}H and ^{32}P from [β-^{32}P]-UDP-[6-^{3}H]GlcNAc into glycopeptides with high affinity for Concanavalin A-Sepharose. Characterization of the enzyme reaction products (derived from either endogenous or exogenous acceptors) showed that α-linked GlcNAc 1-phosphate is transferred *en bloc* to the 6 hydroxyl position of mannose in the high mannose oligosaccharides of the acceptor glycoproteins. This membrane-associated transferase was neither inhibited by tunicamycin, nor stimulated by dolichol phosphate, indicating that the reaction does not proceed *via* a dolichol pyrophosphoryl-N-acetylglucosamine intermediate (Reitman and Kornfeld, 1981).

We propose that the sequential action of the two enzymes described above results in the generation of the phosphomannosyl recognition marker of lysosomal enzymes that is involved in the targetting of these proteins to the lysosomes. In this scheme (see figure 1) the newly synthesized lysosomal enzyme is glycosylated by transfer of the lipid-linked oligosaccharide to the nascent polypeptide chain (structure 1). This oligosaccharide is then phosphorylated by the transfer of N-acetylglucosamine-1-phosphate from the donor, UDP-GlcNAc. Each oligosaccharide thus acquires one or two phosphate residues (the commonest of the many isomers thus generated is shown in structure 2). The outer blocking N-acetylglucosamine residues are then removed to generate structure 3, the mature phosphomannosyl recognition marker. This marker is then involved in the targetting of the lysosomal enzyme to its ultimate destination, in the lysosome.

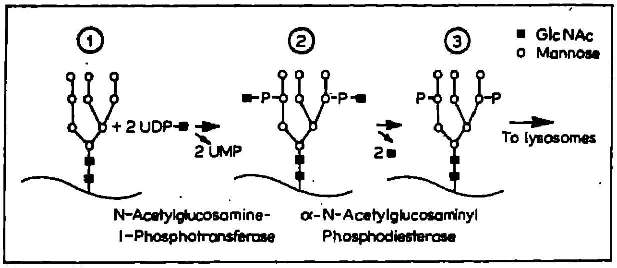
①
②
③
■ GlcNAc
○ Mannose
+ 2 UDP-■
2 UMP
■-P
-P-■
P
-P
2■
To lysosomes
N-Acetylglucosamine-1-Phosphotransferase
α-N-Acetylglucosaminyl Phosphodiesterase

Figure 1. Mechanism for the phosphorylation of mannose residues of lysosomal enzyme oligosaccharides.

I-cell disease and pseudo-Hurler polydystrophy (Mucolipidoses II and III) are autosomal recessive lysosomal storage disorders. Both diseases are characterized by decreased intracellular activities of many lysosomal enzymes, and by markedly elevated levels of the same enzymes in the body fluids (McKusik *et al.*, 1978). Previous studies suggested that these abnormalities might arise from deficient phosphorylation of lysosomal enzymes (Bach *et al.*, 1979; Hasilik and Neufeld, 1980). We therefore studied the generation of the phosphomannosyl recognition marker in fibroblasts from patients with these diseases. The N-acetylglucosaminylphosphotransferase activity (using endogenous acceptors) in cultures of fibroblasts from six normal subjects ranged from 0.67 to 1.46 *p*mol N-acetylglucosamine 1-phosphate transferred/mg protein per h, whereas five pseudo-Hurler polydystrophy and five I-cell cultures transferred less than 0.02 *p*mol/mg protein per h. The activity in five other pseudo-Hurler cultures ranged from 0.02 to 0.27 *p*mol transferred/mg protein per h. The activity of the α-N-acetylglucosaminylphosphodiesterase was normal or elevated in all the I-cell and pseudo-Hurler cultures. Thus, the deficiency of the first enzyme in the pathway for the generation of the phosphomannosyl recognition marker can explain the biochemical abnormalities previously described in these diseases (Reitman *et al.*, 1981).

Note:

Many similar findings have been independently reported by K. von Figura, A. Hasilik, A. Waheed, and others (see references Hasilik *et al.*, 1980; Waheed *et al.*, 1981a, b; Hasilik *et al.*, 1981, and their accompanying papers).

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Some physicochemical aspects of oligosaccharide binding to concanavalin A and wheat germ agglutinin

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Abstract. The binding of fluorescently labelled carbohydrates to concanavalin A and wheat germ agglutinin was studied at equilibrium and by the stopped-flow and temperature-jump relaxation methods. Ligands were mainly the 4-methylumbelliferyl glycosides of α (1→2)-linked manno-oligosaccharides and of β (1→4)-linked chito-oligosaccharides as limited homologous series. They offer distinct advantages, particularly for kinetic studies.

Enthalpic and kinetic considerations suggest that concanavalin A specifically binds a single mannopyranosyl group in α (1→2)-linked manno-oligosaccharides. This occurs preferentially at the non-reducing end. Glycosylation of a carbohydrate with *e.g.* an aryl group does not affect the binding kinetics and for all carbohydrates the association rate is comparable but relatively slow, which indicates that a common process is involved in the binding of all carbohydrates to concanavalin A. The affinity of a carbohydrate for concanavalin A is determined by the dissociation-rate parameter, resulting in a longer residence time for a better ligand.

Interaction of chito-oligosaccharides with wheat germ agglutinin is complex. With the larger members of the 4-methylumbelliferyl chito-oligosaccharides, binding studies were only possible at low fractional saturation to avoid formation of unsoluble complexes. The binding kinetics of wheat germ agglutinin are faster than with concanavalin A and are consistent with a wheat germ agglutinin binding region composed of two adjacent subsites. For binding of the monoside as well as the bioside, two consistent kinetic models apply. They have common that for each ligand there exist two complexes with comparable population.

Keywords. Concanavalin A; wheat germ agglutinin; fluorescence; difference absorption; temperature-jump; stopped-flow.

Introduction

In 1973, Dean and Homer reported that the fluorescence of 4-methylumbelliferyl α-D-mannopyranoside (MeUmb-Man*p*) is totally quenched upon binding to concanavalin A (Con A). Since then, MeUmb-glycosides have emerged as interesting ligands for equilibrium and kinetic studies of carbohydrate-protein interactions. Examples are the studies with the lectins Con A (Loontiens *et al*, 1977a, b; Clegg *et al*, 1977, 1981; Van Landschoot *et al*, 1978a, 1980a, b; Fitos *et*

Abbreviations used: MeUmb-Man*p*- 4-methylumbelliferyl α-D-mannopyranoside; Con A, concanavalin A; WGA, wheat germ agglutinin; Me-Man*p*, methyl-α-D-mannopyranoside.

al., 1979; Farina and Wilkins, 1980), wheat germ agglutinin (WGA) (Privat *et al.*, 1974b; Van Landschoot *et al.*, 1977, 1978b; Clegg *et al.*, 1980; Erni *et al.*, 1980) and those from the seeds of *Ricinus communis* (Khan *et al.*, 1980), *Momordica charantia* (Khan *et al.*, 1981a), *Abrus precatorius* (Khan *et al.*, 1981b), *Bandeiraea* (*Griffonia*) *simplicifolia* (De Boeck *et al.*, 1981), *Pisum sativum* (De Boeck, 1980), *Erythrina cristagalli* (unpublished; in collaboration with J. Iglesias, H. Lis and N. Sharon). Other systems include GlcNAc-directed antibodies (Kieda *et al.*, 1977), lysozyme (Delmotte *et al.*, 1975; Yang and Hamaguchi, 1980a, b) and a β-xylosidase from *Bacillus pumilus* (Claeyssens and De Bruyne, 1978).

This interest in MeUmb-glycosides is due to their versatility in such binding studies. Their large absorption coefficient (about 1.3×10^4 M^{-1} cm^{-1} at 316 to 318 nm) and their fluorescence (maximal emission at 373 nm) allow very sensitive detection. Upon binding to a protein, these ligands often show pronounced difference absorption spectra (in the 300 to 350 nm range) as well as changes in ligand fluorescence properties. In many cases there is a drastic or total quenching of the fluorescence intensity (with lysozyme, an increase is observed). When no quenching occurs, or when it is incomplete, binding can be followed by the substantial increase in ligand fluorescence polarisation (Van Landschoot *et al.*, 1978a; Khan *et al.*, 1980a, b; De Boeck *et al.*, 1981; unpublished results with *Erythrina cristagalli*). All these spectroscopic changes are intense, and were found to be specifically inhibited by the appropriate carbohydrates. Such MeUmb derivatives are particularly valuable for binding studies where nonchromophoric carbohydrates cause little, if any, change in the intrinsic optical properties of a carbohydrate-binding protein such as *e.g.* Con A. In addition, MeUmb-glycosides have been used to study the binding equilibrium (Dean and Homer, 1973; Van Landschoot *et al.*, 1980a) and kinetics (Clegg *et al.*, 1981) of nonchromophoric carbohydrates to Con A. Similarly, MeUmb-glycosides are sensitive indicators of the reactivation of the lectins when the appropriate metal ions are added to a demetallized lectin such as Con A (Harrington and Wilkins, 1978; Christie *et al.*, 1978, 1979; Urdea *et al.*, 1979; De Clercq *et al.*, 1980) or to a mixture of demetallized isolectins I from *Bandeiraea* (*Griffonia*) *simplicifolia* (Harrington *et al.*, 1981).

Here we summarize our results on the binding equilibria and kinetics of α(1→2)-linked manno-oligosaccharides [$(Manp)_n$] with Con A; and of chito-oligosaccharides, [$(GlcNacp)_n$] with WGA. In addition to these ligands, we have mainly used the MeUmb $(Manp)_n$ α-glycosides and MeUmb $(GlcNacp)_n$ β-glycosides with $n = 1$, 2 or 3. For the latter lectin-bound glycosides, the sign or in intensity of their difference absorption spectra, as well as the degree of ligand-fluorescence quenching, reflect the average surrounding of the MeUmb group on these lectins. The binding characteristics of the parent carbohydrates were determined from substitution titrations with a MeUmb-glycoside as a fluorescent indicator ligand (Van Landschoot *et al.*, 1980a). The binding kinetics of the MeUmb-glycosides were studied by the temperature-jump relaxation technique (Clegg *et al.*, 1977, 1980; Loontiens *et al.*, 1977b; Van Landschoot *et al.*, 1980b) and stopped-flow method (Clegg *et al.*, 1977; Van Landschoot *et al.*, 1980b), for methyl α-D-mannopyranoside (Me-Man*p*) a competitive temperature-jump relaxation method was used (Clegg *et al.*, 1981).

Results

Con A*

Equilibrium studies with Con A: The equilibrium binding experiments have provided information on the affinity and on the spectroscopic changes which are involved in Con A-carbohydrate interactions. Low molecular weight carbohydrates like MeUmb-Man*p* and MeUmb $(\mathrm{Man}p)_2$ bind to one site per protomer on Con A, and do so in an independent fashion, both in its dimeric and tetrameric forms. This was shown by equilibrium dialysis, absorption difference spectra titrations (Loontiens *et al.*, 1977a; Van Landschoot *et al.*, 1978a, 1980b) and is very apparent from the Scatchard plot in figure 1, which is obtained from titrations using the total fluorescence quenching of MeUmb-Man*p* when bound to Con A. Interaction of the lectin with these simple carbohydrate derivatives in solution does not show any of the complex binding phenomena which have been reported for membrane bound cell-wall receptors. These have been suggested (Wright, 1980b) to be due to the aggregation of Con A (and WGA) at the cell surface which is dependent upon the concentration of the lectin as well as the carbohydrate. A summary of the binding characteristics for $(\mathrm{Man}p)_n$ and MeUmb $(\mathrm{Man}p)_n$ is listed in table 1 (Van Landschoot *et al.*, 1978a). The preliminary assignment of the binding configuration shown with table 1 is suggested by the optical–signal changes. The value of the change in enthalpy (ΔH°) for all ligands with $\alpha(1\rightarrow2)$ mannosidic linkages is independent of the number of Man*p* groups, or of the presence of a MeUmb group. Since hydrogen bonds, involved in the binding of carbohydrates have a much larger contribution in ΔH than nonspecific interactions, this would imply that Con A specifically binds a single Man*p* group in $\alpha(1\rightarrow2)$-linked manno-oligosaccharides**. The value of ΔH° for Me-Man*p* in table 1 has been confirmed by calorimetric measurements at comparable ionic strength (Munske *et al.*, 1978). The close relation between ΔH° and the carbohydrate-binding affinity is illustrated by comparing the poorer ligand Me α-Glc*p*.($\Delta H^\circ = -18$ kJmol^{-1})† with Me-Man*p* ($\Delta H^\circ = -38$ kJmol^{-1}) (Clegg *et al.*, 1981).

Kinetic studies with Con A: All of the equilibrium experiments have been interpreted assuming a single binding configuration between the carbohydrates and the protein. This is probably not valid for the oligosaccharides containing more than one Man*p* group. Furthermore, from the equilibrium binding studies it is difficult to obtain information whether the carbohydrate binding site in each protomer can simultaneously interact with more than one Man*p* group. On the contrary, the

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* All equilibrium and kinetic data for binding of carbohydrates and Con A were obtained with the fully metellized lectin (Ni^{2+}, Ca^{2+}) that was virtually free of nicked polypeptide chains. The conditions were: 1 M NaCl at pH 5.5 (dimer) or at pH 7.2 (tetramer) in a temperature range of 4 to 40°C. All results are expressed on the basis of Con A protomer with M_r equal to 25,500.

** The conclusion for these ligands can result from steric hindrance that does not occur upon binding of oligosaccharides derived from N-glycopeptides (J. Carver, communicated).

† These values were obtained by simulating and concentration dependence of the ΔH-sensitive amplitudes in competition temperature-jump relaxations with MeUmb-Man*p* as a fluorescent kinetic indicator (Clegg *et al.*, 1981). The ratio of these values for the two Me-glycosides was confirmed by calorimetry under different conditions of pH and at low ionic strength (Dani *et al.*, 1981).

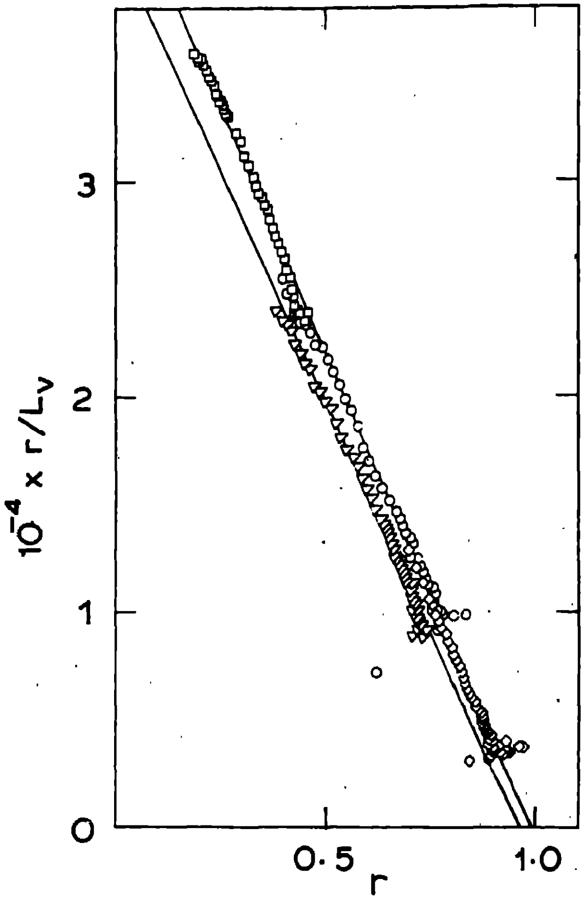

Figure 1. Scatchard plots for the binding of MeUmb-Man*p* to Con A at pH 5.5 and 15.8°C (De Boeck, 1980). The right-hand graph is for linearized titrations acquired by observing the fluorescence quenching of MeUmb-Man*p* during the addition of native Con A to 554 µl of 20.6 µM (□), 87.4 µM (○) and 275 µM (◇) MeUmb-Man*p*, contained in a thermostated 0.5×0.5×4.5-cm cuvette equipped with a motor driven stirrer; 0 to 200 µl of 793 µM native Con A was added from a microburette. Excitation of fluorescence was in the 335 to 358 nm range, with absorbance <0.02 and emission was measured at 373 to 380 nm. All titration data were corrected for a blank titration without MeUmb-Man*p* as well as for dilution; they correspond to $K = (4.51 \pm 0.02)\ 10^4\ M^{-1}$ with 0.985 ± 0.005 binding sites per protomer. The data of the left-hand graph were obtained from a single titration experiment with 87.4 µM MeUmb-Man*p* and a sample of Con A (606 µM) that had first been demetallized, and then reactivated with an excess of Ni^{2+} and Ca^{2+}. These plots show that the binding properties of native, and of reactivated Con A are practically identical.

above data do not indicate the existence of an extended binding region composed of several subsites. By extending the studies into the time domain, we have obtained information about multiple binding possibilities. The following experiments with α(1→2)-linked manno-oligosaccharide derivatives are consistent with a single binding site per protomer of Con A. It interacts with only one Man*p* group at a time, but it can interact differently with any, out of several Man*p* groups in these oligosaccharides (see scheme II of table 2).

Table 1. Characteristics for the binding of α (1→2)-linked MeUmb $(Manp)_n$, $(Manp)_n$ and Me-Man*p* to Con A at 25°C.

	pH	$10^3 \times \Delta\varepsilon_{334}$ $(M^{-1}\,cm^{-1})$	F_∞/F_0	$10^{-4} \times K$ (M^{-1})	$-\Delta H°$ $(kJmol^{-1})$	$-\Delta S°$ $(Jmol^{-1}\,K^{-1})$
MeUmb-Man*p*	5.5	−2.21	0.00±0.02	3.36±0.04	34.8±0.4	30± 1
MeUmb-Man*p*	7.2	−2.21	0.01±0.02	4.26±0.07	35.6±0.8	31± 3
MeUmb-$(Manp)_2$	7.2	+ 1.04	0.34±0.01	13.9 ±0.5	36.0±0.8	22± 3
MeUmb $(Manp)_3$	7.2	+ 0.44	0.40±0.01	8.2 ±0.3	36.8±0.8	29± 4
Me-Man*p*	5.5	–	–	0.64±0.05	38 ±2	56± 7
$(Manp)_2$	5.5	–	–	1.2 ±0.1	33 ±4	32±14
$(Manp)_3$	5.5	–	–	11.0 ±0.5	37 ±2	31± 7

For binding of MeUmb $(Manp)_n$, $\Delta\varepsilon_{334}$ is the change in the molecular extinction coefficient at 334 nm. F_∞/F_0 is the fluorescence of the complex relative to the fluorescence of its free ligand. The data for MeUmb $(Manp)_n$ were obtained by fluorescence titrations at six to ten temperatures and by difference absorption measurements at 334 nm; they are consistent with the results obtained by equilibrium dialysis (Loontiens *et al.*, 1977a; Van Landschoot *et al.*, 1978a, 1980b). The data for the nonchromophoric compounds were obtained from substitution titrations with MeUmb-Man*p* as a fluorescent indicator ligand; the corresponding Scatchard plots were linear provided that a slowly establishing equilibrium, *e.g.* with $(Manp)_2$, was taken into account (Van Landschoot *et al.*, 1980a).

Preliminary binding scheme:

      A   X   Y
      M – F
      M – M – F
M – M – M – F   (Scheme I)
     (M – M – M – F)

X and Y represent distances from site A with M—Man*p* and F—MeUmb. This scheme will be refined into scheme (II) (table 2) in view of the kinetic results.

Table 2. Reaction-rate parameters and signal changes per complex for binding of MeUmb (Man*p*)$_2$ to Con A at 20°C and pH 5.5 according to two consistent mechanisms.

Sliding (sequential)		Alternating (excluded site)	
$A+B \underset{-1}{\overset{+1}{\rightleftharpoons}} C_1 \underset{-2}{\overset{+2}{\rightleftharpoons}} C_2$		$C_a \underset{-a}{\overset{+a}{\rightleftharpoons}} A+B \underset{-b}{\overset{+b}{\rightleftharpoons}} C_a$	
k_{+1}	$= 6.4\times10^4\ M^{-1}\ sec^{-1}$	k_{+a}	$= 3.6\times10^4\ M^{-1}\ sec^{-1}$
k_{-1}	$= 5.7\ sec^{-1}$	k_{+a}	$= 10.1\ sec^{-a}$
k_{+2}	$= 4.3\ sec^{-1}$	k_{+b}	$= 2.8\times10^4\ M^{-1}\ sec^{-1}$
k_{-2}	$= 0.2\ sec^{-1}$	k_{-b}	$= 0.12\ sec^{-1}$
K_1	$= k_{+1}/k_{-1} = 1.1\times10^4\ M^{-1}$	K_a	$= k_{+a}/k_{-a} = 3.5\times10^3\ M^{-1}$
K_2	$= k_{+2}/k_{-2} = 21$	K_b	$= k_b/k_{-b} = 2.4\times10^5\ M^{-1}$
K_I	$= K_1(1+K_2) = 2.5\times10^5 M^{-1}$	K_{II}	$= K_a+K_b = 2.5\times10^5\ M^{-1}$
$F_\infty/F_0\,(C_1)$	$= 0.33$	$F_\infty/F_0\,(C_a)$	$= 0.31$
$F_\infty/F_0\,(C_2)$	$= 0.35$	$F_\infty/F_0\,(C_b)$	$= 0.35$
$\Delta\varepsilon_1(C_1)$	$= 570\ M^{-1}\ cm^{-1}$	$\Delta\varepsilon_1(C_a)$	$= 210\ M^{-1}\ cm^{-1}$
$\Delta\varepsilon_2(C_2)$	$= 1070\ M^{-1}\ cm^{-1}$	$\Delta\varepsilon_2(C_b)$	$= 1040\ M^{-1}cm^{-1}$

The data were obtained by stopped-flow kinetics measuring changes in ligand fluorescence [0.1 and 1 μM MeUmb (Man*p*)$_2$] and ligand absorption [21 μM MeUmb (Man*p*)$_2$] using more than twenty different concentrations of Con A (6 to 340 μM) in excess under pseudo first-order conditions (Van Landschoot *et al*, 1980b). The stopped-flow traces showed a faster apparent reaction-rate constant (110 to 37 m sec) with a saturable amplitude and a slower one (3.9 to 0.3 sec) with a maximal amplitude at 30 μM. The reaction-rate parameters were calculated from linear plots of the sum and of the product of the apparent reaction-rate constants *versus* the sum of the free concentrations of ligand and protein (Bernasconi, 1976). The values for the signal change per complex (F_∞/F_0 and $\Delta\varepsilon$, as defined in table 1) differ for each mechanism. The signal changes for C_2 or C_b are almost identical to those at equilibrium (table 1) due to the preferred binding of this complex *via* its terminal non-reducing Man*p* as in scheme (I). The value of the kinetically defined association constant ($2.5\times10^5\ M^{-1}$) is in fair agreement with the equilibrium value ($1.8\times10^5\ M^{-1}$) at 20 °C.

Binding scheme:

$$\begin{array}{cccccccl} & & A & & X & & Y & \\ M & - & M & - & F & & & C_a \text{ or } C_1 \\ & & M & - & M & - & F & C_b \text{ or } C_2 \end{array} \qquad \text{(Scheme II)}$$

with abbreviations as for scheme (I).

If a monosaccharide were to bind to a lectin with an extended binding region composed of subsites, the number of binding possibilities could equal the number of these subsites. It is therefore relevant that the binding of all monosaccharides that have been investigated with Con A, show a monophasic kinetic process corresponding to a single-step association (Gray and Glew, 1973; Brewer *et al*, 1973, 1974; Lewis *et al*, 1976; Clegg *et al*, 1977, 1981; Loontiens *et al*, 1977b; Williams *et al*, 1978b, 1981; Farina *et al*, 1980). This indicates that the binding region contains but one Man*p*-specific subsite. A detailed temperature-jump relaxation study (Clegg *et al.*, 1977; Loontiens *et al.*, 1977b) with MeUmb-Man*p* and dimeric or tetrameric Con A, showed a single relaxation of the ligand fluorescence or absorption which is related to ligand binding. The dependence of the relaxation time τ and amplitude on the concentration of ligand or protein is consistent with a bimolecular one-step association over a wide concentration range (upto 400 μM). The activation energy for association is high (63 $kJmol^{-1}$) and the activation volume is positive (16 $ml\ mol^{-1}$) (Fitos *et al.*, 1979). Two minor relaxations of protein fluorescence ($\tau \simeq 10$ m sec and $\tau > 1$ sec) were also observed by temperature-jump and pressure-jump (Elson E. L. unpublished) techniques, but it was established (Clegg *et al.*, 1977), that these were unrelated to the binding of carbohydrates, or of metal ions. In addition, the latter two relaxations are unrelated to the dimer-tetramer equilibrium.

Other evidence that a single Man*p* group is involved in the binding is available from the experiments with the fluorescently labelled manno-oligosaccharides. In particular, the complexity of the kinetic progress curves under first-order conditions increases as the number of the Man*p* increases, *i.e.* the stopped-flow progress curves are mono-, bi- or triphasic for $n = 1, 2$ or 3 of MeUmb $(Manp)_n$ (Van Landschoot *et al.*, 1980b).

The kinetic characteristics for MeUmb $(Manp)_2$ binding to Con A are summarized in table 2. The concentration dependencies of the apparent rate constants (a linear increase for the faster process, and a hyperbolic increase for the slower process), and of the corresponding amplitudes are consistent with two nearly indistinguishable mechanisms (Viale, 1971; Van Landschoot *et al.*, 1980b). As shown in table 2, each proposed mechanism involves two complexes with different fluorescence and absorption properties depending on the mechanisms. The first mechanism consists of the bimolecular formation of a complex C_1 that subsequently isomerizes into C_2. This can be interpreted as a sliding movement of MeUmb $(Manp)_2$ on the protein surface. For this mechanism we propose that MeUmb $(Manp)_2$ is initially bound as C_1 through the internal Man*p* at A in scheme (II) and subsequently translocates to form C_2 such that the terminal Man*p* is now bound at A. It is this last step that forms the preferentially bound species at equilibrium. The second mechanism considers the same two bound species (C_1 and C_2 for mechanism I, C_a and C_b for mechanism II), but assumes that a Con A-MeUmb $(Manp)_2$ complex must completely dissociate before binding again in a bimolecular step. The complex C_b is suggested to have its terminal Man*p* bound to site A and, as with scheme (I), this is the more affine complex. However, for scheme (II) the difference in the populations of the two complexes is more pronounced than for scheme (I) (Scheme I, $C_2/C_1 = 21$; scheme II, $C_b/C_a = 68$). The second mechanism (alternating) provides the best opportunity to compare the binding steps for MeUmb $(Manp)_2$ with those for MeUmb-Man*p*, since both kinetic steps of the alternating model are bimolecular. Concerning the two possible positions of the MeUmb group, the one at X for MeUmb $(Manp)_2$, bound as C_a, corresponds best to the MeUmb-Man*p* complex, whereas K_a is about equal to the binding constant for Me-Man*p*. This suggests that the interactions of the MeUmb group with the protein are quite different for MeUmb-Man*p* and MeUmb $(Manp)_2$ and/or that for the latter, the presence of a terminal Man*p* disturbs the specific recognition of the Man*p* group adjacent to the MeUmb group. The two suggested binding mechanisms are interconvertible, and are two extremes of a more general mechanism, which is obtained by inserting an equilibrium converting C_a and C_b in the alternating scheme in table 2. All the results of temperature-jump experiments are consistent with those of the stopped-flow study in table 2.

Stopped-flow traces for the binding of MeUmb (Man*p*)$_3$ to Con A (up to 94 μM) were always triphasic with time constants in the ranges 0.14–0.27 sec, 2.3–0.8 sec and 10–17sec. The fastest process showed the smallest amplitude. The slower phases were difficult to observe by the time available for our temperature-jump technique (<1 sec).

These results are consistent with the recent stopped-flow data which show biphasic progress curves for other "bivalent" carbohydrates such as *p*-NO_2 Phe Man*p* α (1→2) Man*p* and *p*-NO_2 Phe Glc*p* α (1→2) Man*p* but that are monophasic for *p*-NO_2 Phe Gal*p* α (1→2) Man*p* (Williams *et al.*, 1978b, 1981). The latter is not unexpected since simple galactosides do not bind to Con A.

Factors that can increase the affinity of glycosides: From the above kinetic results and in view of the practically constant values of the binding enthalpies in table 1, it appears that all carbohydrates Me-Man*p* (Man*p*)$_n$ and MeUmb (Man*p*)$_n$ bind to Con A with only one Man*p* residue. Yet, the association constants increase markedly within the series (Man*p*)$_n$ as *n* increases ($K_n/K_1 = 1$, 1.9 and 17 for Me-Man*p* and $n = 2$ and 3 in table 1). A statistical reason for this increase in affinity, representing the increase in the possibilities for binding the available Man*p* residues, does not totally account for the largest value. In addition, the results of table 2 indicate a large binding preference for the terminal Man*p* in MeUmb (Man*p*)$_2$. Unspecific interactions could account for these discrepancies and some of these could originate from the aglycon. In addition to the increase in *K* with the MeUmb group (*e.g.* with MeUmb-Man*p* as compared to Me-Man*p*), some *p*-substituted Phe α-Man*p*, with electron releasing substituents bind 4 to 5 times better (Loontiens *et al.*, 1973, 1975) than (Man*p*)$_2$. Such unspecific interactions could also apply to a nonbinding carbohydrate like Gal*p* since *p*-NO_2 Phe Gal*p* α (1→2) Man*p* (Williams *et al.*, 1981) is a better ligand than *p*-NO_2 Phe α-Man*p* or *p*-NO_2 Phe α-2-OMeMan*p* (Williams *et al.*, 1978b, 1981). Unspecific interactions can also occur in *p*-substituted Phe β-glucosides; the corresponding linear free energy relationships with the molecular refractivity of the substituent (Hansch, 1975; Hansch and Calef, 1976) are superior to those with the hydrophobicity parameter (Loontiens *et al.*, 1973). This can arise from interaction with amphiphilic side chains at the surface of a protein.

Conclusions from kinetic studies with Con A: It is interesting to compare the binding kinetics of all the carbohydrates investigated so far; this is done in table 3 together with the recent data of Farina and Wilkins (1980) and Williams *et al.* (1981). The carbohydrates involved are the α and β Me-glycosides of glucose and methyl α-mannoside, arylglycosides and aryldisaccharides. For all these ligands an apparent single-step association is valid; this also holds for a disaccharide if the individual formation of C_a or C_b in table 2 is considered. The association-rate parameters are comparable and low (3×10^4 to 11×10^4 M^{-1} sec^{-1}). This is about four orders of magnitude smaller than can be predicted for a diffusion-controlled process and the high activation energy (63 $kJmol^{-1}$) for binding of MeUmb-Man*p* is also inconsistent with a diffusion-controlled reaction (Clegg *et al.*, 1977).

Table 3. Comparison of the reaction-rate parameters (k_+ and k_-) and kinetically defined association constants (K) for the binding of carbohydrates to Con A acccording to a bimolecular one-step association

	$10^{-4} \times k_{+1}$ (M^{-1} sec^{-1})	k_{-1} (sec^{-1})	$10^{-3} \times K$ (M^{-1})	t (°C)	pH
MeUmb (Man*p*)$_2$^{a}	2.8	0.12	240	20	5.5
	3.6	10.1	3.5	20	5.5
p-NO$_2$ (Man*p*)$_2$^{b}	3.2	0.22	145	25	5.0
	2.3	1.1	21	25	5.0
MeUmb-Man*p*^{c}	10.2	3.2	32	24.1	7.2
	11.3	3.4	33	24.1	5.5
p-NO$_2$ Phe-Man*p*^{d}	5.4	6.2	8.7	25	5.0
p-NO$_2$ Phe-2-O-MeMan*p*^{b}	6.6	8.2	8.1	25	5.0
Me-Man*p*^{e}	4	12	3.3	23.2	7.2
Me-Glc*p*^{e,f}	5	31	1.7	25	7.2; 5.6
MeβGlc*p*^{f}	2.8	400	0.07	25	5.6

[a] Van Landschoot *et al*, 1980b; [b] Williams *et al*, 1978b; [c] Clegg *et al*, 1977; Loontiens *et al*, 1977b; [d] Lewis *et al*, 1976; [e] Clegg *et al*, 1981; [f] Brewer *et al*, 1973, 1974.

Association-rate constants for carbohydrate binding to proteins are usually low (Pecht and Lancet, 1977; Clegg *et al*, 1981) which could be related to the hydrophylic nature of these ligands. For Con A, the comparably slow rate for all ligands points at a common rate -determining step. Theoretically this could arise from a rapid isomerisation in either of the free reaction partners themselves, provided that the concentration of the reacting species were very low; this case is improbable for Con A in view of the controls that were made (Clegg *et al*, 1977). An alternative possibility is a rapid formation of the Con A-carbohydrate complex that is possibly diffusion controlled and consequently undergoes a slow conformational change in the observed time window. This two-step mechanism, for which we could not find any evidence by absorption, fluorescence and polarisation of fluorescence in the μ sec time range, could involve a local change in tertiary protein structure, that comprises hydrogen bond interactions within the protein and with water molecules. Such a conformational change has been postulated from CD (Pflumm *et al*, 1971; Cardin *et al*, 1979) NMR stopped-flow (Grimaldi and Sykes, 1975) and also from infusion of Me-Man*p* in Con A crystals (Hardman and Ainsworth, 1973).

A possible biological consequence can be formulated from the rate parameters in table 3. Since the association-rate parameters are comparable, it is the dissociation-rate parameter that determines the association constant: for a poor ligand like Me-β-Glc*p* dissociation is much faster (400 sec^{-1}) than for a good ligand like MeUmb (Man*p*)$_2$ (with *e.g.* 0.1 sec^{-1}). The slow complex formation, that is characteristic for Con A, as well as the slow dissociation rate of good ligand result in an appreciable lifetime of a complex without imposing the restrictions of a very high affinity. Such a long residence time of an initially formed Con A—receptor complex could be related to the effectiveness for a topological reorganisation when several glycoconjugate receptors interact with a multivalent Con A molecule. A prerequisite for these events to occur would be a sufficiently long lifetime of the initial complex to allow other Con A receptors to form additional interactions by the slow process of lateral diffusion in the membrane.

*WGA**

Equilibrium studies with WGA: The binding of carbohydrates to WGA is more complex than to Con A. It has been shown by X-ray diffraction (Wright, 1980a, b) that in the dimeric WGA with $M_r = 18000$ each protomer possesses a primary and a secondary binding site. Sialic acid binds to the primary site only, whereas $(GlcNAc\textit{p})_2$ binds to both sites. Carbohydrates larger than $(GlcNAc\textit{p})_2$ could not bind to either site in the crystals due to space limitations.

In solution the binding of MeUmb-GlcNAc*p* and WGA, determined by equilibrium dialysis and titrations using difference absorption spectra (Van Landschoot *et al.*, 1977), is simple throughout the saturation range and corresponds to four identical and independent binding sites in the dimeric lectin. These results are consistent with those for reduced $(GlcNAc\textit{p})_4$ (Privat *et al.*, 1974a). Binding studies with MeUmb $(GlcNAc\textit{p})_2$ and MeUmb $(GlcNAc\textit{p})_3$ are only possible at low fractional saturation ($r < 0.4$), probably due to association of lectin molecules yielding insoluble complexes that can be redissolved with an excess of $(GlcNAc\textit{p})_3$. The properties derived from the binding experiments with MeUmb $(GlcNAc\textit{p})_n$ and WGA under these restrictive conditions are given in table 4. Unlike Con A, the value of $-\Delta H°$ for binding of WGA to MeUmb $(GlcNAc\textit{p})_2$ is noticeably larger than for binding of the monosaccharide. The fluorescence of both ligands is totally quenched when they bind to WGA and this, together with the partial quenching for MeUmb $(GlcNAc\textit{p})_3$ fluorescence, suggests a preliminary binding scheme (III) given with table 4. However, the different values of $\Delta\varepsilon$ for the similar difference absorption spectra obtained with MeUmb-GlcNAc*p* and MeUmb $(GlcNAc\textit{p})_2$, given in table 4, suggest that this scheme is too simple (see table 5).

The relative values of the association constants for MeUmb $(GlcNAc\textit{p})_n$ are difficult to compare with those of $(GlcNAc\textit{p})_n$ since the increase in affinity with *n* for the latter ligands varies from 1, 6.5 and 29 (Privat *et al.*, 1974c) to 1, 800 and 3000 (Allen *et al.*, 1973) among the reported values obtained by different methods (Lotan and Sharon, 1973; Nagata and Burger, 1974; Goldstein *et al.*, 1975; Bhavanandan *et al.*, 1979; Peters *et al.*, 1979). This could be due to experimental problems caused by a complex binding phenomenon which is suggested by the concave Scatchard plot for the binding of $(GlcNAc\textit{p})_3$ and WGA isolectin I (Allen *et al.*, 1973), in figure 2. Such conclave plots were also obtained with MeβGlcNAc*p* and $(GlcNAc\textit{p})_2$ and were independent of whether individual isolectins or a mixture of isolectins were used. When 50% displacement of MeUmb $(GlcNac\textit{p})_2$ was taken as an indication for the affinities of Me-βGlcNAc*p*, $(GlcNAc\textit{p})_2$ and $(GlcNAc\textit{p})_3$, these relative values were 1, 15 to 20 and 110 to 120 at 14 to 18°C (Van

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* All equilibrium and kinetic data for binding of carbohydrates to WGA were obtained using 0.1 M NaAc/Hac, 0.5 M NaCl at pH 4.7 between 10 and 40° C. WGA concentrations were expresed as sites on the basis of an equivalent M_r equal to 9000.

Table 4. Characteristics for the binding of MeUmb $(GlcNAcp)_n$ to WGA at 25°C.

	$10^3 \times \Delta\varepsilon_{318}$ $(M^{-1}\ cm^{-1})$	F_∞/F_0	$10^{-4} \times K$ (M^{-1})	$-\Delta H^\circ$ $(kJmol^{-1})$	$-\Delta S^\circ$ $(Jmol^{-1}\ K^{-1})$
MeUmb-GlcNAc*p*	−2.77	−0.01 ±0.02	2.0 ± 0.1	34.3 ± 1.7	32.7 ± 6.3
MeUmb $(GlcNAcp)_2$	−1.07	−0.01 ±0.02	7.5 ± 0.3	41.9 ± 1.3	46.9 ± 4.2
MeUmb $(GlcNAcp)_3$	0	0.56 ±0.02	11.7 ± 0.6	35.2 ± 1.3	20.1 ± 5.0

Δε refers to the change in absorption at 10.8°C for difference absorption spectra that were similar in form for MeUmb-GlcNAc*p* and MeUmb $(GlcNAcp)_2$. The values of F_∞/F_0 and the binding parameters were obtained from fluorescence titrations with an excess of WGA at nine to eleven temperatures. For MeUmb-GlcNAc*p* these results were consistent with those obtained by equilibrium dialysis and by titrations using the ligand difference absorption spectra (Van Landschoot *et al*, 1977).

Preliminary binding scheme:

A B C D
G − F
G − G − F (Scheme III)
G − G − G − F
with G = GlcNAc and F = MeUmb.

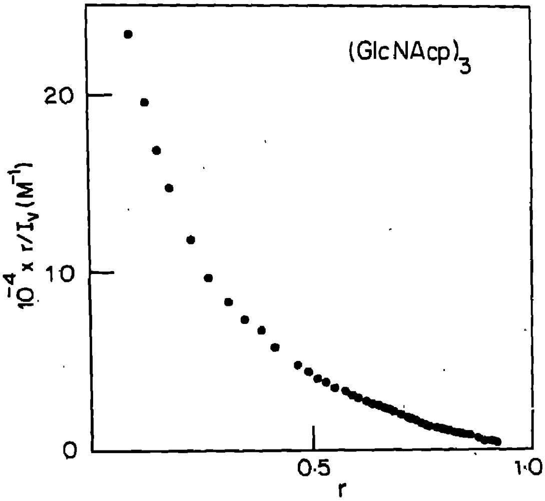

Figure 2. Scatchard plot for binding of $(GlcNacp)_3$ to WGA at pH 4.7 and 16°C. The data were obtained by a continuous substitution titration according to Van Landschoot *et al.* (1980a) using MeUmb $(GlcNAcp)_2$ as a fluorescent indicator ligand. To 2.948 ml of a mixture of 3.24 μM MeUmb $(GlcNAcp)_2$ and 29.1 μM WGA (isolectin I) in a 1×1×4.5-cm cuvette, 3.13 mM $(GlcNacp)_3$ was added from a microburette under continuous stirring. The fluorescence of MeUmb $(GlcNAcp)_2$, released from its totally quenched WGA complex was recorded. After correction for dilution, and for a blank without MeUmb $(GlcNAcp)_2$, the data were transformed into a Scatchard plot. An attempted fit for two different and independent binding sites was unsatisfactory (Van Landschoot, 1978).

Table 5. Reaction-rate parameters and characteristics for binding of MeUmb-GlcNAc*p* and MeUmb (GlcNAc*p*)$_2$ to WGA at 23.6° C and pH 4.7 according to two consistent mechanisms.

Sliding (sequential)	Alternating (excluded site)
$A+B \underset{\substack{-1\\ \text{fast}}}{\overset{+1}{\rightleftharpoons}} C_1 \underset{\substack{-2\\ \text{slow}}}{\overset{+2}{\rightleftharpoons}} C_2$	$C_a \underset{\substack{-a\\ \text{fast}}}{\overset{+a}{\leftrightharpoons}} A+B \underset{\substack{-b\\ \text{slow}}}{\overset{+b}{\rightleftharpoons}} C_b$
MeUmb-GlcNAc*p*: two relaxations (coupled)	
$k_{+1} = 3.0\times10^6\ M^{-1}\ sec^{-1}$	$k_{+a} = 1.5\times10^6\ M^{-1}\ sec^{-1}$
$k_{-1} = 1.8\times10^2\ secsec^{-1}$	$k_{-a} = 3.2\times10^2\ sec^{-1}$
$k_{+2} = 1.1\times10^2\ sec^{-1}$	$k_{+b} = 1.5\times10^6\ M^{-1}\ sec^{-1}$
$k_{-2} = 70\ sec^{-1}$	$k_{-b} = 39\ sec^{-1}$
$K_1 = k_{+1}/k_{-1} = 1.7\times10^4\ M^{-1}$	$K_a = k_{+a}/k_{-a} = 4.7\times10^3\ M^{-1}$
$K_2 = k_{+2}/k_{-2} = 1.6$	$K_b = k_{+b}/k_{-b} = 3.8\times10^4\ M^{-1}$
$K_{\mathrm{I}} = K_1(1+K_2) = 4.3\times10^4 M^{-1}$	$K_{\mathrm{II}} = K_a + K_b = 4.3\times10^4\ M^{-1}$
MeUmb (GlcNAc*p*)$_2$: two relaxations (uncoupled)	
$k_{+1} = 1.0\times10^7\ M^{-1}\ sec^{-1}$	$k_{+a} = 9.3\times10^6\ M^{-1}\ sec^{-1}$
$k_{-1} = 2.0\times10^2\ sec^{-1}$	$k_{-a} = 2.3\times10^2\ sec^{-1}$
$k_{+2} = 21\ sec^{-1}$	$k_{+b} = 1.1\times10^6\ M^{-1}\ sec^{-1}$
$k_{-2} = 18\ sec^{-1}$	$k_{-b} = 16\ sec^{-1}$
$K_1 = 5\times10^4\ M^{-1}$	$K_a = 4.0\times10^4\ M^{-1}$
$K_2 = 1.17$	$K_b = 6.9\times10^4\ M^{-1}$
$K_{\mathrm{I}} = K_1(1+K_2) = 1.1\times10^5 M^{-1}$	$K_{\mathrm{II}} = K_a + K_b = 1.1\times10^5\ M^{-1}$
$\Delta\varphi_2 = 0$ (assumptions)	$\Delta\varphi_a = \Delta\varphi_b$
$\Delta H_1 = -33\ kJmol^{-1}$	$\Delta H_a = -36.8\ kJmol^{-1}$
$\Delta H_2 = -8.1\ kJmol^{-1}$	$\Delta H_b = -37.5\ kJmol^{-1}$
$\Delta H_{\mathrm{I}} = \Delta H_1 + \dfrac{K_2}{1+K_2}\Delta H_2 = -37.3\ kJmol^{-1}$	$\Delta H_{\mathrm{II}} = (K_a\Delta H_a + K_b\Delta H_b)\times (K_a+K_b)^{-1} = -37.3\ kJmol^{-1}$

The data were obtained by the temperature-jump relaxation technique at 20.4+3.2° C measuring the ligand fluorescence. The kinetic data were obtained under the following conditions: 2.1 μM MeUmb-GlcNAc with 5 to 240 μM WGA or 2.6 μM MeUmb (GlcNAc*p*)$_2$ with 2 to 80 μM WGA. The relaxation curves were always bi-exponential. For MeUmb-GlcNAc*p* the faster relaxation (with relaxation times of 3.0 to 1.6 m sec) showed a maximal amplitude at about 25 μM WGA and the slower relaxation (19 to 3 m sec) had a maximal amplitude at about 100 μM WGA. The results with MeUmb (GlcNAc*p*)$_2$ were similar: the faster relaxation (4 to 1 m sec) had its maximal amplitude at 6 μM WGA and the slower one (55 to 10 m sec) was maximal at about 14 μM WGA. The reaction-rate parameters were calculated from linear plots of the sum of the product of the inverse relaxation times *vs* the free concentration of ligand and protein. For MeUmb-GlcNAc*p*, the concentration dependence of the relaxation times was analyzed for kinetically coupled steps 1 and 2. For MeUmb (GlcNAc*p*)$_2$ an analysis for uncoupled steps was completely satisfactory and this property facilitated the calculation of ΔH values from the relative amplitudes (the kinetic change in fluorescence divided by the static fluorescence). It was assumed that ligand fluorescence φ was totally quenched in all complexes. ΔH_1, and ΔH_a were obtained from the slope of a linear plot for the relative amplitude of the faster process *versus* its relaxation time; ΔH_{I} and ΔH_{II}, the kinetically defined overall binding enthalpy, was obtained from the slope of a linear plot for the total relative amplitude *versus* the product of the two relaxation times. For MeUmb (GlcNac*p*)$_2$ the kinetically defined association constant ($1.1\times10^5\ M^{-1}$) and binding enthalpy ($-37.3\ kJmol^{-1}$) were in fair agreement with the values $8.0\times10^4\ M^{-1}$ (at 23.6°C) and $-41.9\ kJmol^{-1}$ obtained at equilibrium.

Landschoot, 1978). Deviations from a simple binding curve, similar to figure 2 have also been observed by Midoux (1980) for the binding of Me-β $(GlcNAc\textit{p})_2$ and WGA at pH 7.2; as measured by the enhancement of tryptophan fluorescence, the curvature of the Scatchard plots was very pronounced for 5 μM WGA, and gradually disappeared when WGA concentrations were increased to 160 μM.

The apparent affinity of Me-β $(GlcNAc\textit{p})_2$ decreases by a factor larger than 10 as the concentration of WGA increases. These complex binding phenomena have been related to aggregation of WGA (Midoux, 1980) and to a monomer-dimer equilibrium of WGA that depends on its concentration, pH and binding of carbohydrates (Midoux, 1980).

Kinetic studies with WGA: The progress curves of the kinetic experiments obtained with MeUmb $(GlcNAc\textit{p})_n$ and WGA were faster than those for Con A, and could conveniently be studied by the temperature-jump relaxation technique. The results (Van Landschoot *et al.*, 1978b; Clegg *et al.*, 1980, in preparation) are summarized in table 5. Unlike the previously discussed results obtained with Con A, both the mono- and disaccharides MeUmb-GlcNAc*p* or MeUmb $(GlcNAc\textit{p})_2$ show biphasic kinetic processes when binding to WGA. This, together with the values of Δ H° for the binding of the mono- and disaccharides, (table 4) suggests that the binding region contains two subsites in WGA.

The biphasic behaviour of the relaxation times for the binding of WGA to both ligands MeUmb-GlcNAc*p* and MeUmb $(GlcNAc\textit{p})_2$ is similar; the inverse of the relaxation time of the faster process increases linearly with concentration, whereas the slower one increases hyperbolically, approaching a constant value at high concentrations. This behaviour is consistent with the two mathematically equivalent models in table 5. Here, as in the case of Con A, these two models can be regarded as a binding followed by a sliding over the site, or as two alternative bimolecular binding processes. For the latter mechanism, the deduced values of ΔH_a and ΔH_b for MeUmb $(GlcNAc\textit{p})_2$ are virtually the same. In the sliding mechanism however, fast binding in step 1 has a value of $-\Delta H_1$, that is much larger than $-\Delta H_2$ for the slow monomolecular process in step 2. Both complexes C_1 are almost equally populated at equilibrium. This value of $-\Delta H_2$ for the sliding mechanism nearly accounts for the difference in the equilibrium constants for MeUmb-GlcNAc*p* and MeUmb $(GlcNAc\textit{p})_2$ in table 4; this can be interpreted as the contribution of the second GlcNAc*p* residue to the equilibrium value of ΔH for MeUmb $(GlcNAc\textit{p})_2$. An attempted refinement of scheme (III) is represented in figure 3. This was done considering the signal changes in table 4 and the values of

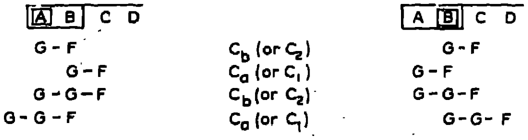

Figure 3. Suggested possible binding positions of MeUmb $(GlcNAc\textit{p})_n$ $[G_n F]$ at a binding region of WGA composed of two subsites A and B with different affinities for GlcNAc*p*. Left: the binding region is "open" at the left of A, the affinity at A is larger than at B, sites B and C cause 100% quenching of the MeUmb group F; at B, the value of $-\Delta\varepsilon$ is larger than at C. Right: the binding region at the left of A is not accessible to G, the affinity at B is larger than at A, sites B, C and D cause 100% quenching of group F and the value of $-\Delta\varepsilon$ decreases in the direction of B, C, D

the association constants per step in table 5. It should be noted that the two possibilities in figure 3 differ in the position of the subsite with the highest affinity. It is difficult as yet to compare our results for MeUmb (GlcNAc*p*)$_r$ with those from X-ray diffraction data for (GlcNAc*p*)$_n$ (Wright, 1980b) in view of the different binding properties of these two ligand series, both in the crystals and in solution.

The binding kinetics of MeUmb (GlcNAc*p*)$_3$ and WGA showed a total of four relaxations of fluorescence. The two faster ones had positive amplitudes similar to the two ligands discussed above and the amplitude of the fastest one became unobservable for concentrations to WGA larger than 10 μM. The values and the concentration dependencies of these two relaxation times were similar to those for MeUmb-GlcNAc*p* and MeUmb (GlcNAc*p*)$_2$. At concentrations higher than 10 μM however, two additional relaxations appeared. An analysis of the relaxation times shows that the simple alternative binding mechanism is not valid for MeUmb (GlcNAc*p*)$_3$. It should still be established if these complex kinetics of MeUmb (GlcNAc*p*)$_3$ are related to the impaired accessibility of the binding region of WGA for the higher members of the chito-oligosaccharides derivatives (Wright, 1980b), or to higher aggregations of the protein-oligosaccharide complexes.

Concluding remarks

The combination of equilibrium and kinetic measurements with Con A and WGA have resulted in highly informative studies of the carbohydrate-protein interactions. The intense signal changes observed with the fluorescent MeUmb-glycosides of the simple sugars and of the low molecular oligosaccharides are advantageous for these experiments, especially the kinetic studies. For all α (1 → 2)-linked manno-oligosacchrides, Con A binds a single Man*p* residue, whereas for chito-oligosaccharides, WGA binds two adjacent GlcNAc*p* residues. For all carbohydrates that bind to Con A, there seems to be a common, rate-determining association step, resulting in similar and characteristically low association rates, whereas the slow but variable dissociaton rates determine the association constants. These two slow rates are probably a favourable factor for the biological action of these lectins when one considers the slow rearrangements in the membrane leading to multiple cell-wall receptor-lectin complexes.

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Note added in proof

The quenching of MeUmb-Man*p* fluorescence upon binding of this ligand to Con A has been further exploited as an indicator system to study binding of the required metal ions [Koenig, S. H., Brown, R. D. III, Brewer, C. D. and Sherry, A. D. (1982) *Biochem. Biophys. Res. Commun.*, **109**, 1047–1053; Sophianopoulos, A. J. and Sophianopoulos, J. A. (1983) *Arch. Biochem. Biophys.*, **223**, 350] and to investigate carbohydrate binding to monomeric Con A [Sophianopoulos, A. J. and

Sophianopoulos, J. A. (1982) *Arch. Biochem. Biophys.*, **217,** 751–754]. We have recently published a more complete analysis of the kinetic data on WGA [Clegg, R. M., Loontiens, F. G., Sharon, N. and Jovin, T. M. (1983) *Biochemistry*, **22,** 4797–4804]. As to the versatility of 4-methylumbelliferyl glycosides in binding studies with lectins, MeUmb difference absorption spectrometry seems to be of general applicability since it enables monitoring of binding with all of the ten lectins investigated to date [De Boeck, H., Lis, H., Sharon, N. and Loontiens, F. G. (1983) *15th FEBS Meeting Brussels (Belgium)*, July 24–29, p. 213]. In this respect we have reported on a continuous titration technique of differences absorption [De Boeck, H., Loontiens, F. G. and De Bruyne, C. K. (1982) *Analyt. Biochem.*, **124,** 308–313]. Detailed studies on the binding of MeUmb Galβ (1→3) GalNAc to the lectin from peanut have dealt with aspects of equilibrium [Decastel, M., Tran, A.-T. and Frénoy, J. P. (1982) *Biochem. Biophys. Res. Commun.*, **106,** 638–643; De Boeck, H., Matta, K. L., Claeyssens, M., Sharon, N. and Loontiens, F. G. (1983) *Eur. J. Biochem.*, **131,** 453–460] and stopped-flow kinetics [Loontiens, F. G. (1983) *FEBS Lett.*, **162,** 193–196]. Similar approaches of lectin-carbohydrate interaction with the lectin from soybean [De Boeck, H., Loontiens, F. G., Lis, H. and Sharon, N. (1983) *15th FEBS Meeting Brussels (Belgium)*, July 24–29, p. 213] will be published in detail or are reaching a final stage with the lectin from *Erythrina cristagalli.* With cellulolytic enzymes, 4-methylumbelliferyl cello-oligosaccharides are useful to investigate the degradative mechanisms [Van Tilbeurgh, H., Claeyssens, M. and De Bruyne, C. K., *FEBS Lett.*, **149,** 152–156].

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Haemolysis of rabbit erythrocytes by a lectin from the seeds of *Croton tiglium*

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Abstract. The kinetics of haemolysis of rabbit erythrocytes by *Croton tiglium* lectin was studied as a function of concentration of the lectin and erythrocytes. The length of the prelytic period decreased with increasing lectin concentrations, indicating that the secondary events at the membrane which follow the binding of the lectin to cell surface carbohydrate receptors are accelerated at higher surface concentrations of the lectin. The rate or extent of haemolysis was not affected by the inclusion of ions like K^+, Ca^{2+} and Mg^{2+} in the medium or by the substitution of ionic medium by a non-ionic medium. The inhibition of haemagglutination and haemolysis of rabbit red cells by *Croton tiglium* lectin by antilectin rabbit serum was observed. A possible mechanism of haemolysis by the lectin is discussed.

Keywords. Lectin; haemolysis; *Croton tiglium*.

Introduction

Proteins with haemolytic activity, or in general with cytolytic activity, have been of much interest to membrane biologists, since the study of the mode of action of such cytolytic agents has contributed considerably to the understanding of complexities of biomembrane organization and function (Alouf, 1977). Erythrocytes, the most well understood of all animal cells, have served as a convenient tool for the assay of lytic potency of cytolytic agents as well as for the elucidation of lytic mechanism. The intracellular composition of animal red cells differs markedly from that of the surrounding milieu. Thus, they exist in a steady state far from thermodynamic equilibrium and their viability depends on the ability of the erythrocyte membrane to selectively include certain components and exclude others (Sachs *et al*, 1975). Haemolytic proteins bind or enzymatically degrade specific membrane components like lipid or protein, thereby affecting membrane organization in such a way that it fails to serve as a selective permeability barrier. There is intrusion of water resulting in swelling and the erythrocyte is transformed from its normal discocyte form to a taut sphere. The cell ultimately bursts with the leakage of the cytoplasmic material into the medium. Although there is wide diversity in the pathways of haemolysis induced by various cytolytic proteins, the colloid osmotic swelling is in most cases the last step. Bacterial haemolysins like staphylococcal α-toxin, staphylococcal β-toxin and thiol-active haemolysins like streptolysin *O*, which are among the more well-known haemolysins, illustrate the diversity in haemolytic mechanisms. Staphylococcal α-toxin, a surface-active protein induces haemolysis by interfering with the hydrophobic region of the membrane, staphylococcal β-toxin induces lysis by virtue of its phospholipase *C* activity specific for sphingomyelin, and streptolysin *O* by disorganizing membrane structure by binding specifically to cholesterol. However, there is usually a series of membrane events, more or less obscure, which precede haemolysis. The study of these events, which intervene the primary process of interaction of cytolytic agents with specific membrane components and the final loss of permeability properties, is expected to throw light on the question of how protein and lipid are woven into a structure which imparts the membrane physical stability and biological function.

All hitherto known cytolytic proteins have been isolated from either animal or bacterial sources. Although the presence of haemagglutinating and haemolytic proteins in the seed extract of *Croton tiglium* (Fam. *Euphorbiaceae*) has been known for a long time (Osborne, 1924; Karrer *et al.*, 1925; Stirpe *et al.*, 1976; Lin and Huang, 1976), the activities have been purified only recently. Banerjee and Sen (1981) reported the purification of a lectin from *C. tiglium* seeds with haemagglutinating activity towards erythrocytes of sheep, cow and a few other animals and haemagglutinating as well as haemolytic activity towards rabbit erythrocytes. The lectin purified by ion-exchange chromatogrphy and gel filtration, was found homogeneous by polyacrylamide gel electrophoresis at pH 4.5 and at pH 8.3 and also by immunodiffusion and immunoelectrophoresis. That the haemagglutinating and haemolytic activities were associated with the same protein was demonstrated by the co-purification of the two activities, the simultaneous loss of the two activities on heating and on exposure to acid and alkaline pH values, and also by the ability of sheep erythrocytes to adsorb the haemolytic activity towards rabbit red cells. Although the lectin was not inhibited by simple sugars, both haemagglutination and haemolysis were inhibited by trypsin released glycopeptides from sheep red cell surface and pronase-digested trypsin fragments, suggesting that binding of the lectin molecule to carbohydrate receptors on rabbit erythrocytes surface was involved in inducing both haemagglutination and haemolysis. The carbohydrate nature of the receptor of *C. tiglium* lectin was further confirmed by the marked decrease in sensitivity of rabbit red cells pre-treated with D-galactose-binding lectins like *Abrus precatorius* agglutinin and *Ricinus communis* agglutinin towards *C. tiglium* lectin induced haemolysis. These results demonstrated that the primary processes in haemagglutination and haemolysis were associated with the specific binding of *C. tiglium* lectin molecules with rabbit erythrocyte carbohydrate receptors. However, the differential sensitivity of trypsinized rabbit erythrocytes to *C. tiglium* lectin-induced haemagglutination and haemolysis as well as different effects of temperature on the two phenomena, suggested that the secondary membrane events leading to agglutination and lysis followed different pathways. It will be interesting to know how the binding of *C. tiglium* lectin molecules to cell surface glycoprotein or glycolipid receptors triggers off events which lead to lysis.

In the present communication, we present the kinetics of haemolysis as a function of lectin concentration and red cell concentration and also the effect of changing milieu composition on haemolysis. The inhibition of haemolytic and haemagglutinating activities of *C. tiglium* lectin by rabbit antiserum is also discussed in this paper. These information may lead to a better understanding of secondary membrane events.

Materials and methods

Preparation of protein solutions

The lectin was isolated and purified from *C. tiglium* seeds as described by Banerjee and Sen (1981). The concentration of the purified lectin was determined spectrophotometrically at 280 nm using a value of $E^{1\%}_{1\,\mathrm{cm}} = 15.6$ dl g^{-1}.

Purification of IgG from rabbit antiserum and preparation of Fab fragment

The isolation of IgG from rabbit antiserum against purified *C. tiglium* lectin was carried out according to Fahey and Terry (1967). Twenty five ml of rabbit antiserum dialysed against 0.015 M potassium phosphate buffer, pH 8.0 was applied on a column (28 × 2.8 cm) of DEAE-cellulose equilibrated with the same buffer. Fractions which eluted out during the washing of the column with the starting buffer contained purified IgG with antilectin antibody activity and were pooled and precipitated by $(NH_4)_2SO_4$ at 80% saturation.

The degradation of IgG to Fab fragment was carried out according to Porter (1959). Ten ml of IgG solution containing 150 mg protein in 0.1 M sodium phosphate buffer, pH 7.0 containing 0.01 M cysteine and 2 mM EDTA were incubated with 1.5 mg papain. After incubation, the reaction mixture was exhaustively dialysed against water during which the F_c fragment crystallized out. The clear solution obtained after centrifugation of the dialysate, was subjected to chromatography on Sephadex G-200 when the Fab fragment emerged as a large symmetrical peak.

Assay of haemolytic activity

This was carried out according to Kabat and Mayer (1967). Rabbit erythrocytes washed 3–4 times with 0.01 M sodium phosphate buffer containing 0.15 M NaCl, pH 7.0 were made into an approximately 6% suspension (v/v) or in any other isotonic buffered medium such as 0.3 M D-glucose or 0.3 M sorbitol buffered with 0.01 M sodium phosphate, pH 7.0. The suspension was filtered through a thin bed of adsorbent cotton to remove any clumps and diluted with phosphate buffered saline so that the final suspension on complete lysis with 20 vol. of distilled water gave an absorbance of 0.60 at 541 nm. This corresponds to a 6% suspension (v/v). Five millimeters of this suspension was incubated at 25° C with an equal volume of lectin solution containing 0.2–10 μg of lectin. Aliquots of 0.2 ml of reaction mixture were withdrawn at definite time intervals, diluted to 2 ml with chilled PBS and centrifuged immediately in the cold. The absorbance of the supernatant was read at 541 nm against a blank without lectin. Haemolytic activity was expressed as percentage of red cells lysed under the conditions of assay.

Results

Kinetics of haemolysis of rabbit erythrocytes as a function of lectin concentration

Figure 1 shows the time-course of haemolysis of a 3% suspension of rabbit erythrocytes as a function of different lectin concentrations in the range of 0.1–5 μg/ml. It will be noted from the figure 1 that the 'haemolysis *vs.* time' course is sigmoidal and is characterized by three distinct regions. These are, (i) a prelytic period or

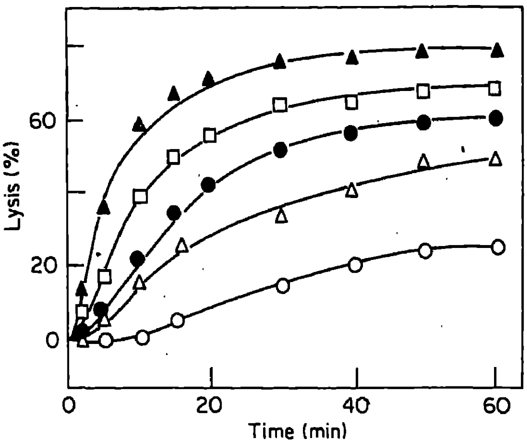

Figure 1. Kinetics of haemolysis of a 3% suspension of rabbit erythrocytes in 0.01 M sodium phosphate buffer containing 0.15 M NaCl (PBS) at different concentrations of *C. tiglium* lectin at 25°C. (○), Per cent haemolysis at 0.1 μg/ml; (△), 0.5 μg/ml; (●), 1 μg/ml; (□), 2 μg/ml and (▲), 5 μg/ml lectin concentration.

lag phase during which there is relatively little or no haemolysis, (ii) a period of rapid haemolysis during which there is almost a linear relation between per cent haemolysis and time and (iii) a static phase during which there is no further lysis. The sigmoidal nature of the curve indicates that the binding of the lectin molecule to rabbit erythrocyte surface and the final act of haemolysis are intervened by a series of membrane processes. The kinetics of the secondary membrane processes appear to be reflected in the length of the prelytic period. It will be seen from figure 1 that the length of the lag phase which is hardly detectable at lectin concentrations above 1 μg/ml, decreases with increasing lectin concentration. This suggests that the secondary membrane processes are accelerated at higher concentrations of the lectin molecules on the rabbit erythrocyte surface.

Kinetics of haemolysis of rabbit erythrocytes as a function of erythrocyte concentration

Figure 2 shows the time-course of haemolysis of rabbit erythrocytes at a lectin concentration of 1 μg/ml at different erythrocyte concentrations in the range 2–10%. Figure 2(A) shows the plot of absorbance at 541 nm, which is a direct measure of the number of cells lysed against time, while figure 2(B) shows the rate of per cent haemolysis, a normalized quantity which takes into account the total number of cells present. It will be seen from figure 2(A) that almost the same number of cells are lysed during the first five min, irrespective of the total number of cells present. A low cell surface density of lectin molecules resulting from the presence of a large number of red cells competing for a given amount of the lectin delays the onset of lysis, and this explains the observed insensitivity of the initial rate of per cent haemolysis to the number of cells present. It will be further noted that the rate as well as the extent of per cent haemolysis is maximum at 2% red cell concentration and minimum at 10% concentration. This is due to the fact that there is a larger percentage of red cells with the optimum number of lectin molecules bound on them, when a relatively fewer of the latter are present.

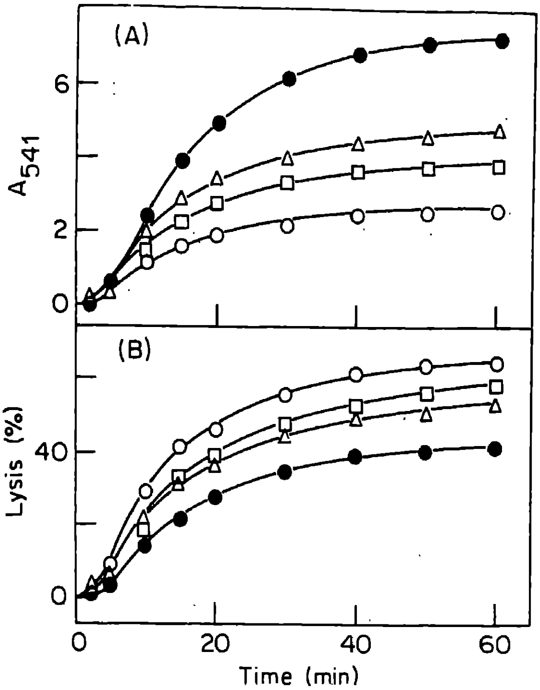

Figure 2. Kinetics of haemolysis of rabbit erythrocytes at a lectin concentration of 1 μg/ml at different erythrocyte concentrations, (A) shows the plot of absorbance at 541 nm, and (B) shows per cent haemolysis. (○), Absorbance and per cent haemolysis at 2% erythrocyte concentration; (□), 3% concentration; (Δ), 5% concentration; (●), 10% erythrocyte concentration.

Inhibition of haemagglutination of sheep erythrocytes and haemolysis of rabbit erythrocytes by rabbit antilectin antiserum and Fab from antilectin IgG

Table 1 shows the effect of preincubation of *C. tiglium* lectin solution with rabbit serum and antilectin rabbit serum on its haemagglutinating and haemolytic activities. It will be noted that the haemolytic activity of *C. tiglium* lectin towards rabbit

Table 1. Inhibition of *C. tiglium* lectin-induced haemagglutination of sheep erythrocytes and haemolysis of rabbit erythrocytes by normal rabbit serum, antilectin antiserum and Fab fragment.

	Dilution or concentration completely inhibiting 4 minimum haemagglutinating dose	Dilution or concentration completely inhibiting haemolysis of rabbit erythrocytes by lectin at a concentration of 1 μg/ml[a]
Normal serum	No inhibition with undiluted serum	No inhibition with undiluted serum
Antilectin antiserum	1 : 8	1 : 10
Fab fragment from antilectin rabbit IgG	200 μg/ml	200 μg/ml

[a] Per cent haemolysis of a 3% suspension of rabbit erythrocytes was monitored after 10 min of incubation.

red cells monitored after 10 min was almost totally inhibited by rabbit antiserum at a dilution of 1 : 10, while the haemagglutinating activity towards sheep red cells is inhibited at a dilution of 1 : 8. No effect was observed with serum from non-immunized animals. To exclude the possibility that the inhibition of haemolysis and haemagglutination arose as a result of the removal of the lectin from the solution by the formation of insoluble antigen-antibody complex and was thus only apparent, experiments were done with the Fab fragment of the antilectin antibody. Table 1 shows that the monovalent antibody, Fab fragment, is also potent in inhibiting haemagglutinating and haemolytic activity.

Since it has been shown that the haemagglutinating and haemolytic activities of the lectin are primarily associated with the carbohydrate-binding function, it appears that at least some of the antibody-combining sites of the lectin are either the same with, or are in close proximity to the sugar-binding site of the lectin.

Effect of EDTA, ions K^+, Ca^{2+} and Mg^{2+} on the kinetics of haemolysis

Figure 3 shows that the substitution of 0.01 M sodium phosphate in PBS by 0.01 M Tris-HCl, pH 7.0, the incorporation of 10 mM Ca^{2+}, Mg^{2+}, or K^+ or 1 mM EDTA did not affect the kinetics of haemolysis. The results indicate that divalent cations like Ca^{2+}, Mg^{2+} are not implicated in either the binding of the lectin molecule to cell surface or in the secondary membrane events.

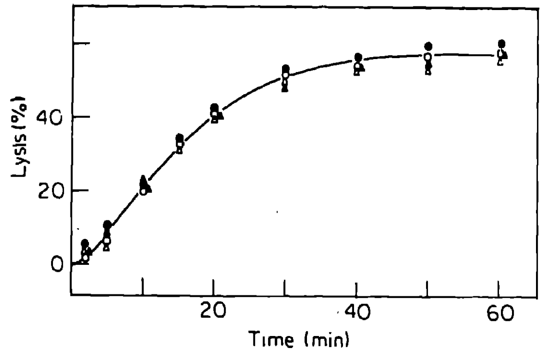

Figure 3. Effect of changing ionic composition on the rate and extent of haemolysis of rabbit erythrocytes at a lectin concentration of 1 μg/ml of 25° C. (○), Per cent haemolysis of rabbit erythrocytes in PBS; (●), 0.01 M Tris-HCl buffer, containing 0.15 M NaCl and 0.01 M $CaCl_2$, pH 7.0; (Δ), 0.01 M Tris-HCl buffer containing 0.15 M NaCl and 0.01 M $MgCL_2$, pH 7.0; (▲), 0.01 M Tris-HCl buffer containing 0.15 M NaCl and 0.001 M EDTA, pH 7.0.

Effect of non-ionic environment of the rate of haemolysis

Figure 4 shows the haemolysis of a 3% suspension of rabbit erythrocytes by *C. tiglium* lectin at a concentration of 1 μg/ml at 25° C in isotonic-D-glucose and sorbitol buffered with 0.01 M sodium phosphate buffer pH 7.0. It will be noted that haemolysis is almost completely inhibited in isotonic glucose solution. However, there was no change in the minimum haemagglutinating dose. Furthermore, if rabbit erythrocytes in isotonic glucose were incubated for 10 min with *C. tiglium* lectin at a concentration of 1 μg/ml and subsequently washed and resuspended in PBS, haemolysis took place. This showed that while haemolysis was

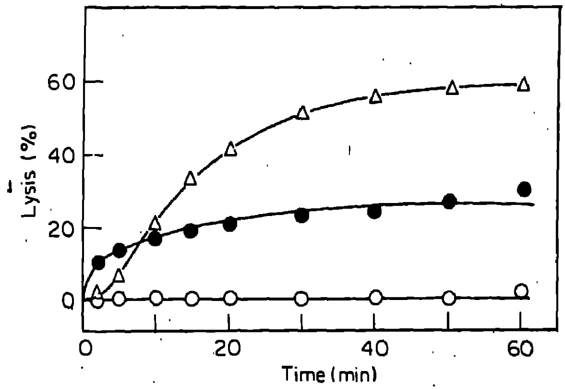

Figure 4. Per cent haemolysis of rabbit erythrocytes at 1 μg/ml lectin concentration in non-ionic environment. (○), Per cent haemolysis of rabbit erythrocytes incubated in 0.30 M D-glucose buffered with 0.01 M sodium phosphate, pH 7.0; (Δ), 0.30 M sorbitol solution buffered with 0.01 M sodium phosphate, pH 7.0; (●), per cent lysis of rabbit erythrocytes which were incubated for 10 min at 1 μg/ml lectin concentration in isotonic D-glucose solution washed twice with isotonic D-glucose solution and finally resuspended in PBS.

inhibited in the pesence of 0.1 M glucose, the binding of the lectin molecule to rabbit red cells and their agglutination were not. These results indicate that an ionic environment is not essential for binding and haemagglutination.

The binding of the lectin molecule to rabbit red cell surface might induce changes in membrane permeability and like ionophores render them permeable to cations resulting in an imbalance of osmotic pressure across the erythrocyte membrane. Such a mechanism would preclude haemolysis in a non-ionic milieu. Alternatively, D-glucose which is readily transported across erythrocyte membrane (Nafatlin and Holman, 1977), might inhibit haemolysis by causing increase of viscosity of haemoglobin and thereby preventing its release through membrane lesions (Seeman, 1974). To eliminate one of these two possibilities rabbit erythrocytes were incubated with lectin in isotonic sorbitol, which has no such effect on haemoglobin. Figure 3 shows that there is no significant change either in the extent or kinetics of haemolysis in isotonic sorbitol, implying that ionic environment is not essential for haemolysis.

Further, the absence of lag phase and completion of the process of lysis within 10 min, when cells previously incubated with lectin in D-glucose are exposed to PBS indicate that secondary membrane processes essential to haemolysis proceed uninterrupted in 0.3 M D-glucose. Thus, it is apparent that the observed inhibition of haemolysis in D-glucose is superficial and has no bearing either on the primary event of lectin cell surface interaction or on the secondary membrane processes.

Discussion

In this paper we have presented the results of our studies on the effect of various parameters on the haemolysis of rabbit red cells by *C. tiglium* lectin in continuation of our previous report (Banerjee and Sen, 1981) which described the purification and physicochemical properties of the lectin and also established the involvement of the carbohydrate binding site in inducing both haemagglutination and haemolysis.

Since it was established that *C. tiglium* lectin induces haemolysis by binding to rabbit red cell surface carbohydrate receptors, it is interesting to consider how the interaction of the lectin with cell surface glycoprotein or glycolipid can trigger off events which lead ultimately to cell lysis. Although lectins are known to affect cell surface morphology and to induce complex biochemical events like agglutination and mitosis by binding specifically to carbohydrate receptors (Goldstein and Hayes, 1978), no lectin has been shown to have cytolytic activity. In other words, it is unlikely that the loss of structural integrity of the rabbit red cell membrane results directly from alterations in topography or mobility of membrane proteins induced by binding of the lectin to carbohydrate receptors. The involvement of the secondary membrane events is suggested by the study of the kinetics of haemolysis (figure 1) which indicates that there is a finite time interval (lag phase) between the binding of the lectin molecules and the escape of haemoglobin. Further the length of this lag phase which is a measure of the kinetics of the secondary membrane events yet to be defined, is a function of the concentration of lectin-receptor complex (figures 1 and 2). These secondary events are, however, not identical with those leading to haemagglutination as shown by the differential response of trypsinized rabbit red cells to *C. tiglium* lectin-induced haemagglutination and haemolysis as well as by the different temperature-sensitivity of the two processes (Banerjee and Sen, 1981). Thus, while agglutination follows from cross-linking of adjacent cells through polyvalent lectin molecules, which may be preceded by redistribution of lectin-receptor complexes to form clusters or patches (Inbar and Sachs, 1973), haemolysis follows from altogether different membrane events. In view of these considerations and the requirement of phospholipase or protease activity to induce haemolysis (Alouf, 1977), it is not unlikely that binding of *C. tiglium*, lectin to cell surface glycoprotein might stimulate phospholipase or protease activity. There are reports that certain lectins stimulate enzymatic activity of membrane proteins. The effect may be an indirect one, resulting from lectin-induced membrane perturbations, *e.g.* the stimulation of galactosyl-transferase activity by binding of concanavalin A to Golgi membranes (Young *et al.*, 1976) or may result directly from lectin-glycoprotein interaction as in the case of the stimulation of β-D-galactoside α2→6-sialyltransferase of bovine colostrum by bovine heart lectin (Scudder *et al.*, 1982).

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Plant lectins specific for N-acetyl-β-D-galactosamine

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Abstract. N-Acetyl-D-galactosamine in β-linkage being ubiquitous in cell surface glycoproteins, their interaction with lectins specific for this sugar moiety may be a significant event in cell adhesion phenomena. This article discusses the common β-N-acetyl galactosamine-specific lectins, with particular stress on the lectin from winged beans (*Psophocarpus tetragonolobus*).

Keywords. β-N-Acetyl-D-galactosamine; blood group A; winged bean.

Introduction

In recent years, lectins have been recognised as useful probes for structural investigations of polysaccharides and complex carbohydrates on cell surfaces. Some lectins are highly specific for certain blood groups and thus may be used for blood group typing. Certain lectins differentiate malignant from normal cells (Sharon and Lis, 1972). Various hypotheses have been advanced regarding the natural physiological function of lectins in plants and animals that contain them. Unequivocal proof is now available for the suggestion that lectins of leguminous plants mediate the attachment of nitrogen fixing bacteria to their root nodules (Bohlool and Schmidt, 1974). Empirical findings suggest that animal lectins may be involved in cell–cell adhesion and differentiation (Rosen *et al*, 1973; Yamada *et al*, 1975). The proposed role for lectins in sugar transport is yet to be demonstrated (Brisk and Chrispeals, 1972). Any of these functions however, involves the interaction of lectins with glycoconjugates. Since the latter often contain N-acylated glycosamines investigations on lectins specific for these sugar moieties would be fruitful.

Lectins that specifically bind N-acetyl-D-galactosamine are particularly important since the latter is the determinant sugar moiety in blood group A substance on the erythrocyte surface. The present paper is a discussion on the widely used N-acetyl-D-galactosamine binding lectins.

Soybean (*Glycine max*) lectin

Soybean lectin (SBA) was originally purified by Liener and Pallansch (1952). Later Sharon and his co-workers have purified the same lectin by affinity chromatography on a column of 6-aminocaproyl-β-D-galactosylamine coupled to Sepharose (Gordon *et al*, 1972). They have studied extensively the physicochemical

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Abbreviations used: SBA, Soybean lectin; M_r, molecular weight; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PHA, phytohaemagglutinin; WBA, winged bean agglutinin.

properties of the lectin. The molecular weight (M_r) was found to be 110,000 dalton consisting of four identical subunits of 30,000 ± 500. The protein was therefore a tetramer (Lis and Sharon, 1973). It contains 4.5% D-mannose and 1.2% N-acetyl-D-glucosamine (Lis *et al.*, 1966). The carbohydrate linkage of SBA was through asparagine moiety to N-acetyl-β-D-glucosamine moiety. The carbohydrate-peptide linking group was identified as $(\text{Man})_{4\text{-}5}$ GlcNAc $(\text{Man})_{5\text{-}4}$ $(\text{GlcNAc})_{1\text{-}2}$-ASn. All the mannose residues are α-linked and the N-acetyl-D-glucosamine residues are β-linked. The soybean oil meal was found to contain three minor isolectins which are separable on DEAE-cellulose columns (Yamada *et al.*, 1975).

The agglutination of erythrocytes or malignant tissue culture cells by SBA is strongly and specifically inhibited by GalNAc (0.05–0.1 μmol/ml) or by disaccharide with GalNAc at their non-reducing end and to a lesser degree by Gal (Lis *et al.*, 1970). SBA contains two identical binding sites for GalNAc per 120,000 dalton with $Ka = 3.0 \times 10^4$/mol.

Lima bean (*Phaseolus lunatus*) lectin

Lima bean lectin was the first plant haemagglutinin shown to exhibit blood group specificity (Boyd and Reguera, 1949). The agglutinating activity resided in two isolectins with the same aminoacid and carbohydrate composition, but the M_r of one (component II, 110,000–138,000) was twice that of the other isolectin (component III, 195,000–269,000) (Gailbraith and Goldstein, 1972a). On sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) with β-mercaptoethanol, both components yielded identical subunits of M_r about 60,000 dalton. It has been suggested that lima bean isolectins are tetramer and dimer of 60,000 dalton disulphide-containing subunits.

These lectins are also glycoproteins, each containing 6–7 mol mannose, 2 mol glucosamine, 1 mol fucose and pentose per 31,000 *g* protein (Gailbraith and Goldstein, 1972a). They also contain Mn^{2+} and Ca^{2+} and the amount of Mn^{2+} is 0.7–11 mol/mol component III and 1.2 mol/mol component II (Gailbraith and Goldstein, 1972b). Lima bean lectins are specific for type A human erythrocytes and N-acetyl-D-galactosamine is specific and reversible inhibitor of the lectins.

Red kidney bean (*Phaseolus vulgaris*) lectin

The term phytohaemagglutinin (PHA) usually refers to a crude preparation from red kidney bean. Crude PHA possessed erythroagglutinin, leucoagglutinin and mitogenic activities. This was the first lectin shown to be mitogenic (Nowell, 1960; Robins, 1964). Several groups have attempted to separate the various activities, but results were often contradictory and somewhat confusing (Lis and Sharon, 1973). The M_r of the lectins, range from 91,000 to 150,000 having 8–4 subunits. Even there was discrepancy in the subunit M_r ranging from 29,000 to 36,000. This confusion can only be settled by further work on the lectins. The lectins are glycoproteins containing mannose and glucosamine, while certain preparation contained fucose, xylose and arabinose, in addition to the above mentioned carbohydrates (Ohtani *et al.*, 1980).

Vicia cracca lectin

Vicia cracca lectin had been isolated by Ruediger (1977) and Karhi and Gahmberg (1980) independently. The methods of affinity chromatography used by the two laboratories are different. Ruediger had used N-acetyl-D-galactosamine coupled to CNBr-activated Sepharose with spacer, while the latter author had used immobilised porcine gastric A/H substance. The physicochemical and biological properties of the isolated lectins were identical. The native lectins had a M_r of 100,000 with a subunit M_r of 29,000. Ruediger (1977) had isolated two isolectins by affinity chromatography. Karhi and Gahmberg (1980) had not mentioned any isolectins. They had shown that the lectin was a glycoprotein contaning 5% carbohydrate with mannose and N-acetyl-glucosamine as the major carbohydrates. It was also shown that the protein contained no sulphur aminoacids and a relatively higher content of aspartic acid. The lectins isolated from both the laboratories were blood group A specific.

Horse gram (*Dolichos biflorus*) lectin

This lectin had been purified to homogeneity on polyleucyl hog AH substance column. It is a glycoprotein with a M_r of 141,000 and had about four subunits. It contained no cysteine. It precipitated blood group A_1 and A_2 substances and other polysaccharides having terminal non-reducing N-acetyl-D-galactosamine residues. Etzler and Kabat (1970) and Etzler (1972) have shown the importance of N-acetyl group in C-2 and the stereochemistry of the hydroxyl on C-4. Recently Etzler and Borrebaeck (1980) have shown the presence of a cross-reacting immunomaterial in the extracts of stem and leaves of the *Dolichos biflorus* plant. The antibody was prepared against the homogeneous lectin from the seeds (Etzler and Borrebaeck, 1980).

Other GalNAc binding proteins of plant origin had been purified from the seeds of *Wistaria floribunda* by Osawa and Toyoshima (1972) as well as from the seeds of *Sophora japonica* by Poretz (Poretz, 1972). These two lectins are also inhibited by other sugars.

Winged bean (*Psophocarpus tetragonolobus*) lectin

In the course of a search for carbohydrate-binding proteins from leguminous seeds, an N-acetyl-D-galactosamine-binding protein from winged bean was purified to homogeneity in this laboratory (Appukuttan and Basu, 1981). The seeds were obtained from locally cultivated beans. The winged bean agglutinin (WBA) was purified by affinity chromatography on N-caproylated galactosamine coupled to Sepharose. Unlike the N-acetyl-D-galactosamine binding lectins described above WBA is not a glycoprotein. Molecular weight determined by gel filtration on Biogel-P 100 and on SDS–PAGE in the presence of 2-mercaptoethanol gave values of 41,000 and 35,000 respectively (figure 1). This lectin like wheat germ agglutinin is a single polypeptide unit containing at least two binding sites for sugar so that it can behave as an agglutinin.

This lectin also binds to galactose though to a lesser extent than N-acetyl-D-galactosamine. Table 1 gives the inhibition capacities of various sugars in agglutination by the lectin.

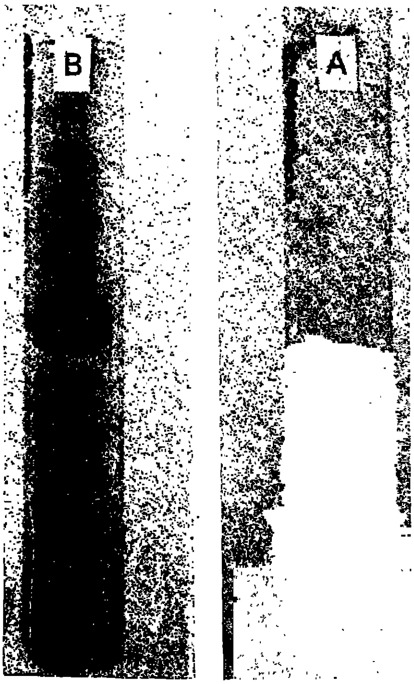

Figure 1. A. Polyacrylamide gel electrophoresis of purified winged bean agglutinin at alkaline pH (8.3). Twenty-five μg was used per tube. **B.** SDS-PAGE of the same protein Details are given in methods. Twenty-five μg protein was used per tube.

Table 1. Inhibition capacities of common sugars on agglutination of erythrocytes by winged bean agglutinin.

Sugar	Minimum concentration (mM) required to inhibit twice the haemagglutinating amount of lectin
Glucose	100
Galactose	12.5
Mannose	N.I.
N-Acetyl-D-glucosamine	100
N-Acetyl-D-galactosamine	1.56
Lactose	6.25
Fucose	N.I.
Galactosamine	N.I.
Ribose	100
D-Fructose	N.I.
α-Methylglucoside	100

Aliquots of 0.2 ml in PBS containing 2 μg protein (double the minimum amount of protein for haemagglutination) with or without serial dilutions of the sugars were incubated for an hour at 25° C and 0.05 ml of 5% suspension of erythrocytes in PBS added. Agglutination was noted after 2 h with occasional shaking.

N.I.—No inhibitory effect even upto 200 mM concentration.

A notable feature of the general affinity chromatographic method of isolation of the N-acetyl-D-galactosamine binding lectins is that the N-caproyl-D-galactosamine group attached to the matrix also binds the lectin. Thus the acyl group need not necessarily be acetyl.

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Electrokinetic studies on concanavalin A as a tool to probe the surface characteristics of differentiated lymphoid cells

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Abstract. The redistribution of surface receptors induced by the binding of concanavalin A to different types of lymphoid cells was studied by the techniques of cell electrophoresis and fluorescence microscopy. The cells studied included, splenic lymphocytes from normal healthy as well as terminally leukaemic mice, thymocytes from mice of varying ages from newborns to adults and antigen sensitised or educated lymphocytes. These cells were in different stages of growth and/or differentiation. The nature and especially the behaviour of surface receptors in response to treatment with concanavalin A under capping conditions differed markedly but appeared to be dependent on the differentiational status of the cells. On this basis, the adult thymocytes were found to consist of two sub-populations differing in their proliferative and differentiational status. The proportions of these varied during their ontogenic development. Lymphocytes specifically committed to an antigen bound concanavalin A but were found to be incapable of bringing about the redistribution of the surface receptor-ligand complexes.

Keywords. Lymphocyte differentiation; concanavalin A; electrophoretic mobility; capping.

Introduction

The lectin concanavalin A (Con A) has proved to be a very useful probe in elucidating differences between the surface architecture of normal and malignant cells (reviewed in Nicolson, 1976; Brown and Hunt, 1978). Its specific mitogenic action has also been employed to assess the functional status of a sub-population of lymphocytes *i.e.* *T*-(thymus derived) cells (Greaves and Janossy, 1972; Stobo *et al.*, 1972). Furthermore, its ability to induce redistribution (patching, capping and endocytosis) of receptor-ligand complexes on cell-surfaces leads to alteration in their surface charge density (Yamada and Yamada, 1973; Wioland *et al.*, 1976; Sainis *et al.*, 1979a) which are measurable by the biophysical technique of cell-electrophoresis. On the basis of the biphasic changes in the electrokinetic behaviour of normal mouse lymphocytes we have clearly demonstrated the existence of two different types of receptor sites for Con A (Sainis *et al.*, 1979a, b; Sainis and Phondke, 1980). These sites differ in their mobility in the plane of the cell membrane and the binding of the lectin to these sites results in quantitatively distinct modulations of electrophoretic mobility of normal splenic lymphocytes.

Abbreviations used: Con A, Concanavalin A; EPM, electrophoretic mobility measurement; FITC-Con A, Fluoresceinated Con A; TRITC-Con A, tetramethyl rhodaine labelled Con A; α-MM, α-methyl mannoside; PNA, peanut agglutinin; DNFB, dinitrofluorobenzene.

On the other hand splenic lymphocytes from spontaneously leukaemic mice of the same AKR strain display, only a single type of receptor sites for the lectin (Sainis *et al.*, 1982). Yet, these sites differ from any of the types on the normal lymphocytes in their response to high concentrations of the lectin (Sainis *et al.*, 1982). Since it has been suggested that the malignant development involves an arrest in cellular differentiation (Nakamura and Metcalf, 1961; Metcalf, 1962), it was interesting to explore whether the Con A—induced modifications of electrophoretic mobilities could serve as a structural probe for studying lymphoid growth and differentiation. In the present investigations we have, therefore, examined the interaction between Con A and lymphoid cells of growing AKR mice and also the antigen sensitised lymphocytes of the adult mice.

Materials and methods

Lymphocytes from spleens, lymph nodes and thymus were obtained from normal healthy AKR mice (8 weeks old) as well as from young mice of various ages. Cell suspensions were prepared in standard saline solution (0.146 M NaCl + 0.01 M KCl, pH 7.2). All electrophoretic mobility (EPM) measurements were made with the cylindrical cell electrophoresis apparatus described by Bangham *et al.* (1958), at 5° C or 25° C (Sundaram *et al.*, 1967). Treatments with Con A were carried out in various schedules as described previously (Sainis *et al.*, 1979a, b). For fluorescence studies, different preparations of the lectin *viz.* fluoresceinated Con A (FITC—Con A) and tetramethyl rhodamine labelled Con A (TRITC—Con A) were prepared according to the method of de Petris (1975).

Results

The responses of the splenic lymphocytes of normal and leukaemic AKR mice to their interaction with Con A provide the basis for subsequent studies on differentiating lymphoid cells. Though the details of these interactions are described elsewhere (Sainis *et al.*, 1979a, b, 1982; Sainis and Phondke, 1980) the salient features may be briefly stated as follows:

Interaction of Con A with normal splenic cells

The treatment of normal lymphocytes with the lectin under capping conditions (*i.e.* 22° C or 37° C for 1 h) results in biphasic changes in their electrophoretic mobility (EPM). These consist of an increase in the EPM at low concentrations of Con A and reduction in the same below that of the untreated cells at higher (≥ 15 μg/ml) concentrations of the lectin. Analysis of this profile clearly established that these changes result from a step-wise, sequential interaction between the lectin and two types of receptor sites on the surface of the same cell (Sainis *et al.*, 1979a, b; Sainis and Phondke, 1980). These receptor-types are:

Type I receptors: These sites are the first to interact with Con A. Binding of the lectin to these sites, by itself, does not alter the electrophoretic mobility. They are induced to undergo redistribution *i.e.*, capping at low concentrations of Con A. This process is accompanied by an enhancement in the cellular EPM (figure 1A).

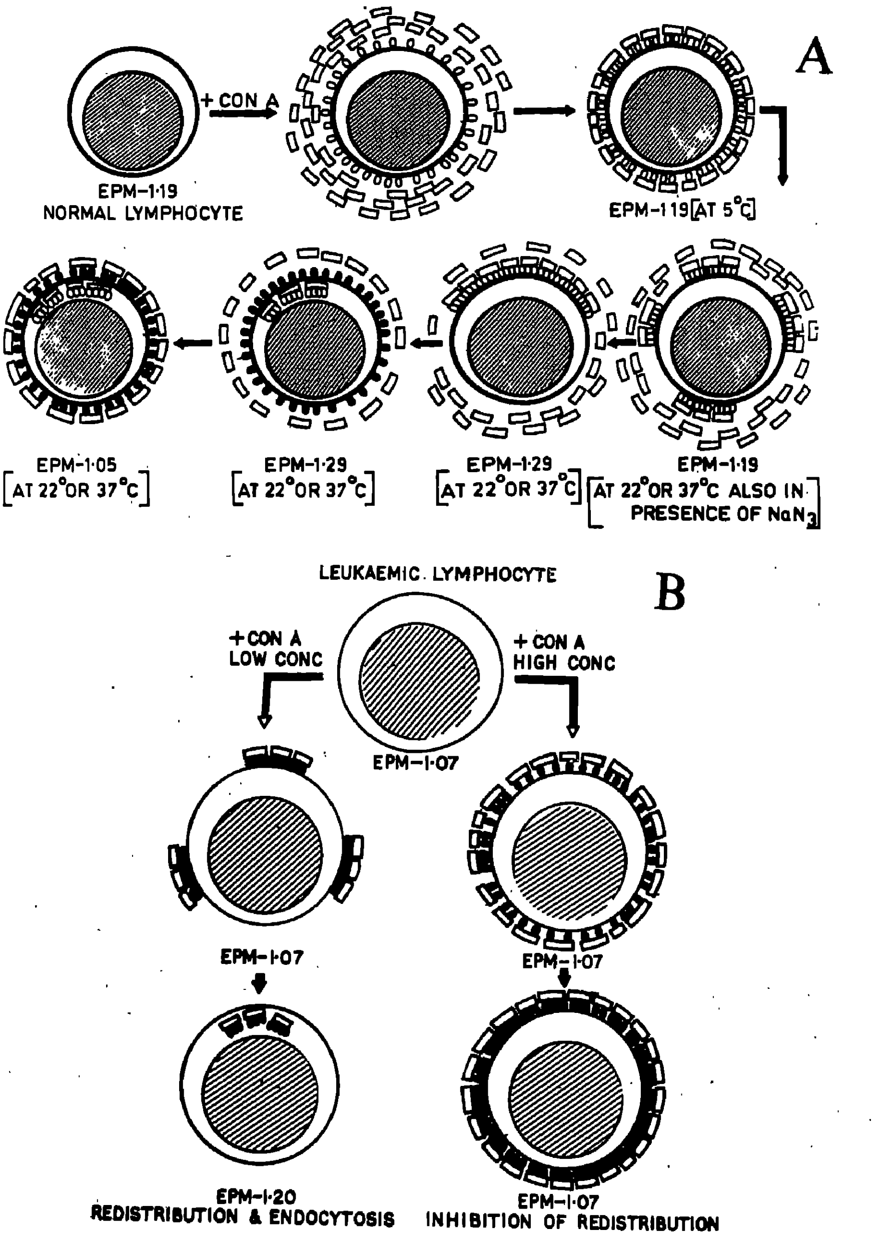

Figure 1. Pictorial representations of the electrophoretic status and the interaction of surface receptors to Con A on lymphocytes of AKR mice.
A. Normal lymphocytes. (□), Con A molecule; (○), type I receptors; (●), type II receptors. **B.** Lymphocytes of leukaemic mice. (□), Con A molecule; (●), receptors for Con A.

Type II receptors: These emerge only as a consequence of the redistribution of receptors of type I. Mere binding of the lectin to these sites reduces the EPM of the lymphocytes below that of the untreated cells. They fail to undergo redistribution (figure 1A). Fluorescence studies clearly show that full endocytosis of type I sites is not necessary for the emergence of the second type of receptors. Even capping or macropatching are sufficient (Sainis and Phondke, 1980).

Using lymphocyte sub-populations separated by their differential adhesion to nylon, it is seen that these two types of receptor sites are present on a subpopulation of the *T*-lymphocytes (Sainis *et al*, 1979a).

Receptors for Con A on leukaemic cells

The essential features of the behaviour of Con A-treated splenic lymphocytes from AKR mice having spontaneous lymphoblastic leukaemia are depicted in figure 1 B. The leukaemic lymphocytes undergo an increase in EPM at low concentrations of the lectin (2–7.5 μg/ml). However, at higher concentrations (≥ 10 μg/ml) their mobility is the same as that of the controls. This is attributed to the inhibition of the laternal mobility of their lectin-binding sites by high concentrations of the lectin (figure 1 B; Sainis *et al*, 1982). Such an inhibition is not observed in the normal cells. Thus the receptors for Con A on the leukaemic lymphocytes are qualitatively distinct from those on the normal cells in their inability to undergo capping at high concentrations of the lectin (Sainis *et al*, 1982). This can ensue from the preponderence of proliferating cells (Blomgren and Andersson, 1969).

Interaction with Con A during lymphoid growth and differentiation

The characteristic differences in the electrokinetic profiles and properties of receptors for the lectin among Con A—treated normal and leukaemic cells prompted us to examine whether in a particular lymphoid tissue, various stages during growth and differentiation could be associated with characteristic profiles of interaction with Con A. Comparative assessment of these interactions was first carried out in the thymocytes of adult and developing AKR mice. Thymus is a very crucial organ in immunological development. It is a seat for cell recruitment and traffic. Stem cells from bone marrow come under the differentiational influence in the thymus before migrating out to the peripheral lymphoid organs as mature T-lymphocytes. There are several reports of cellular heterogeneity within the thymus (Boyse *et al*, 1968; Leckband and Boyse, 1971; Reisner *et al*, 1976; Papiernik *et al*, 1977; Shortman *et al*, 1977).

Interaction of Con A with adult thymocytes:

Figure 2 shows the electrophoretic mobilities of adult thymus cells treated at various concentrations of Con A under capping conditions. The profile was bimodal. There was an initial increase in EPM at 5 μg/ml of Con A. The EPM further increased at moderately high concentrations (15–25 μg/ml) of the lectin. At very high concentrations of Con A, the mean EPM remained unaltered (figure 2).

The close relationship between increase in EPM and the extent of redistribution (Sainis *et al*, 1979a) suggested that at concentrations between 15 and 25 μg/ml of Con A, a higher proportion of thymocytes should be undergoing capping. However, as shown in figure 3, the proportion of thymocytes displaying redistribution progressively decreased with increasing concentration of FITC–Con A. At very high concentrations of the fluorescent lectin very few thymocytes showed capping (figure 3). At no time/no concentration of the lectin more than 40% of the thymocytes showed capping.

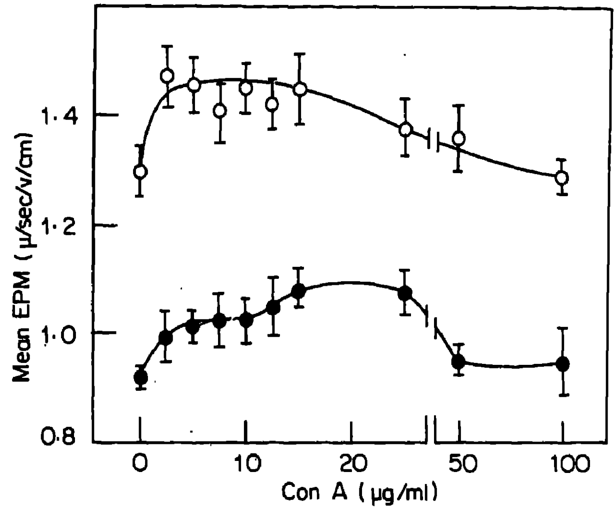

Figure 2. Electrophoretic mobilities of thymocytes and hydrocortisone resistant thymocytes of adult AKR mice after treatment with Con A. 5 × 10^6 cells/ml were incubated with various concentrations of Con A at 22°C for 1 h. They were washed and their electrophoretic mobilities were measured at 25°C in a cylindrical cell microelectrophoresis apparatus in standard saline solution. Each point shows the mean EPM and bars indicate 95% confidence limits.

(●), Adult thymocytes; (○), hydrocortisone resistant thymocytes.

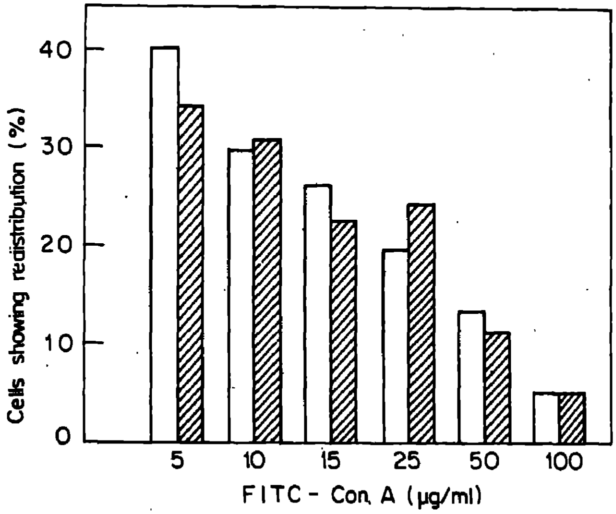

Figure 3. Extent of redistribution of Con A receptors on adult thymus cells and hydrocortisone resistant thymus cells.

5 × 10^6 Cells/ml were incubated with 5 to 100 μg/ml FITC-Con A at 22° C for 1 h. They were washed and fixed with 4% formaldehyde for 20 min. All the cells bound FITC-Con A. Total number of cells showing patching, capping and endocytosis were counted after observation under a Zeiss-epiillumination microscope and expressed as per cent of all thymocytes.

(□), Adult thymocytes; (▨), hydrocortisone resistant thymocytes.

The increase in the EPM of thymocytes was observed to be due to capping induced by the lectin, as it was inhibited by sodium azide. The unmodified EPM and poor proportion of capped cells at very high concentrations was observed throughout the incubation period. It was possible that this was due to the inhibition of redistribution of receptors by high concentrations of the lectin. To resolve this point the thymocytes were first incubated with high concentrations of Con A. When these cells were subsequently treated with optimum concentrations of α-methyl mannoside (α-MM), they showed enhanced proportion of capping and increase in EPM (N. N. Johsi, K. B. Sainis and G. P. Phondke, unpublished observations). Inhibition of receptor redistribution on thymocytes by high concentrations of lectin was thus confirmed. Similar observations were made earlier in leukaemic lymphocytes (Sainis *et al.*, 1982).

The next question concerned with this bimodal electrophoretic profile was whether it represented increasing alteration in the same set of cells or changes in the receptor distribution on different sub-populations of cells. If all the cells capped at a low concentration (5 μg/ml) of Con A and a major fraction of them possessed the second set of receptors which bound Con A post-redistributionally, it could lead to a smaller proportion of cells showing redistribution at moderate concentration of the lectin. Thymocytes were, therefore, pulsed at low as well as at moderate concentrations (25 μg/ml) of FITC Con A at 5° C. The excess of lectin was removed and the cells were allowed to undergo capping (Sainis *et al.*, 1979b). Thirty eight per cent of total thymocytes showed capping on pulse-treatment at 5 μg/ml, while only 28% of the total thymocytes capped when pulsed with FITC Con A at 25 μg/ml (table 1). This compared well with 40% and 20% of cells show-

Table 1. Capping and binding of Con A to second type of receptors in adult thymocytes.

Conc. of FITC-Con A μg/ml	Type of treatment	Cells with FITC-Con A caps[a]	Cells with TRITC-Con A free region[a]	Cells showing second type of receptor[b]
5	Pulse	38%	N.D.	—
5	Direct	40%	35.4	83%
25	Pulse	28%	N.D.	—
25	Direct	20%	1%	5%

[a] Per cent of total thymocytes

[b] Per cent of cells showing FITC-Con A caps.

N.D. Not determined.

Adult thymocytes (5×10^6) were first incubated separately with 5 μg/ml or 25 μg/ml of FITC-Con A at 5° C (Pulse) or at 22° C (direct). Pulsed cells were washed in cold and reincubated at 22° C. After incubation at 22° C for 30 min cells were washed and fixed in formaldehyde. Fixed cells were reincubated with 20 μg/ml of TRITC-Con A for 30 min at 22° C. They were washed and observed in a Zeiss-epiillumination microscope for FITC-Con A caps and the binding of TRITC – Con A to these capped cells, in an area of membrane free of the FITC – Con A cap (to second type of receptor sites as described in ref. Sainis and Phondke, 1980).

ing redistribution after direct incubation with these concentrations of FITC–Con A respectively (figure 3). It, therefore, appeared that the cells undergoing redistribution at low and moderate concentrations of the lectin probably belonged to different sub-populations within the thymus.

Such a possibility implied that the population undergoing redistribution at low concentrations of Con A might possess second set of receptor sites like the normal T-cells. To confirm this possibility thymocytes were first allowed to cap the receptors bound to FITC–Con A at the concentrations of 5 and 25 μg/ml respectively. They were fixed with formaldehyde and were reincubated with a Con A preparation labelled with different fluorochrome (TRITC–Con A; Sainis and Phondke, 1980) and examined. Most of the cells which capped FITC-Con A (5 μg/ml) showed exclusive labelling with TRITC–Con A (>80% of the capped cells). Thus almost all the thymocytes which capped at low concentration of Con A possessed two types of receptors for Con A as in normal T-cells(table 1).

In contrast, very few of the cells which capped at moderate concentrations (25 μg/ml) of FITC-Con A displayed such an exclusive double-labelling, suggesting that they lacked the two types of receptor sites as in normal T-cells (table 1). Thus it appeared that the thymocytes consist of two sub-populations differing in their ability to undergo Con A-induced receptor redistribution. These sub-populations would have the following characteristic features.

Sub-population A: It was induced to cap by low concentrations of Con A but the resultant alteration in EPM was relatively low in magnitude (figure 2). It constituted approximately 40% of the thymus cells and like the normal T-cells displayed the two types of sequentially interacting receptors for the lectin. It is possible that these cells display high affinity for Con A. At very high concentrations of the lectin the lateral mobility of the receptors on this sub-population was inhibited.

Sub-population B: It constituted nearly 20% of thymocytes. These cells probably displayed Con A receptors of low affinity as it underwent redistribution at moderately high concentrations (25 μg/ml) of Con A. The net increase in EPM brought about by this process was, however, higher in magnitude as compared to that in sub-population A. They seemed to possess only a single type of receptors, inhibitable by very high concentrations of the lectin as also observed in the case of the leukaemic lymphocytes (Sainis *et al*, 1982).

Identification of thymocyte sub-populations

In order to relate the above sub-populations with differentiationally distinguishable thymocytes hitherto reported (Boyse *et al*, 1968; Leckband and Boyse, 1971; Reisner *et al*, 1976; Papiernik *et al*, 1977; Shortman *et al*, 1977) these studies were repeated using hydrocortisone resistant thymocytes (Papiernik *et al*, 1977) and thymocyte sub-populations fractionated by agglutination with peanut agglutinin (PNA; Reisner *et al*, 1976). The cortisone resistant cells are considered to be mature cells and resemble the peripheral T-cells, electrophoretically (Wioland *et al*, 1972; Phondke *et al*, 1975). When these cells were treated with Con A under capping conditions, the enhancement in the mean EPM was recorded over a broader range of concentrations of Con A (2.5 to 25 μg/ml) as shown in figure 2. In peripheral T-cells this effect was seen only between 2.5 to 12.5 μg/ml of the lectin (Sainis *et al*, 1979a). Likewise, the redistribution induced by FITC-Con A was observed over a similar range of lectin concentration. The unaltered EPM of these cells at high concentrations of the lectin was attributable to the inhibition of redistribution (N. N. Joshi, K. B Sainis and G. P. Phondke, unpublished observations). Furthermore, since their proportion within the thymus is very small (≃5%, Papiernik *et al*, 1977) they were unlikely to be the major constituent of the sub-populations.

Another possibility could have been that the two sub-populations represented the mature and immature thymocytes as defined by Reisner *et al.* (1976) on the basis of the agglutination brought about by PNA. However, when thymocytes were separated into PNA—agglutinable and non-agglutinable fractions, significant differences in their profiles of Con A—induced redistribution were not seen.

Interaction of Con A with developing thymocytes

Ontogenic development represents an interplay of proliferation and differentiation. The electrokinetic profiles of Con A treated thymus cells from new born (0–18 h age), 9 days old and 21 days old AKR mice are shown in figure 4 as representative examples. The thymocytes from new born mice showed enhancement in EPM at low concentration of Con A (figure 4). At higher concentration the mean EPM was marginally reduced. In contrast, thymocytes of 9 days old and 21 days old mice showed typically bimodal profiles as a function of lectin concentration (figure 4). It is possible that the two peaks in these profiles emanate from the interaction of the lectin with the two sub-populations in the thymus.

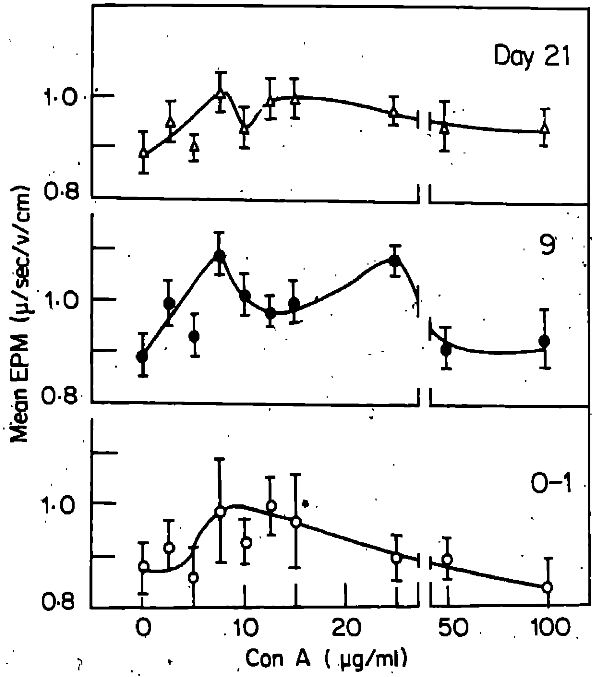

Figure 4. Electrophoretic mobilities of Con A—treated thymocytes from developing AKR mice (0–21 days old). Cells were treated under capping conditions and EPM were measured at 25°C. Each point shows mean EPM and the bars show 95% confidence limits.

Thymocytes from: (○), 0–1 day old mice; (●), 9 days old mice and (△), 21 days old mice.

Interaction of Con A with lymphocytes following antigen-induced differentiation

Though peripheral T and B lymphocytes represent terminal stages of normal lymphoid ontogeny, these cells can further proliferate and differentiate in response to an antigenic stimulus. To study the effect of Con A on the electrokinetic properties of antigen specific differentiated cells, AKR mice were immunised with sheep erythrocytes (5×10^8 SRBC *i.p.*) or contact sensitized to dinitrofluorobenzene (DNFB, Phanupak *et al.*, 1974). The immune lymphocytes were treated with Con A in the same way as the normal lymphocytes. In either case biphasic changes in the EPM were observed like those in normal lymphocytes (figure 5). Only the magnitudes of change in EPM at a given concentration of the lectin were different in the normal and immune lymphocytes (figure 5). The proportions of cells undergoing capping were also similar (table 2).

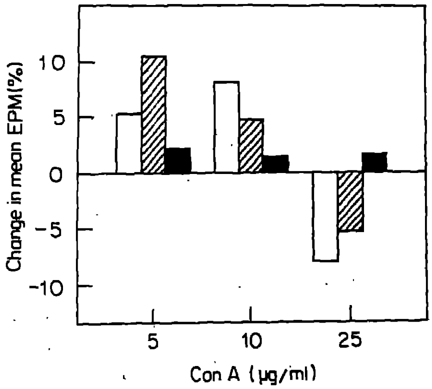

Figure 5. Extent of change in the electrophoretic mobilities of normal lymphocytes, immunised lymphocytes and educated T-cells following interaction with Con A under capping conditions. Change in EPM was calculated at each of the concentrations of the lectin and expressed as per cent of the EPM of untreated cells.

(□), Normal splenic lymphocytes; (▨), splenic lymphocyte of mice immunised with sheep erythrocytes (primary response); (■), educated T-cells.

Table 2. Redistribution of Con A receptors on antigen—stimulated lymphocytes.

Cell type	Cells showing redistribution (%)
Unimmunised lymphocytes	34
SRBC immunised lymphocytes	30
DNFB sensitised lymphocytes	30
Educated T-cells	8

Normal and immune lymphocytes were pulsed with 50 µg/ml of FITC-Con A at 5° C and reincubated at 22° C for 30 min. This treatment is equivalent of direct incubation at low concentrations of Con A (Sainis *et al.*, 1979b). It also eliminates the effects of post-redistributional binding of the lectin to the second type of receptor sites.

It was possible that due to the small number of specifically differentiated cells in the spleen or lymph nodes of the immunised mice, the effect of Con A on these cells was marked by the changes in the larger population of antigenically uncommitted cells. This point was validated by the electrophoretic and fluorescence measurements on a population of lymphocytes enriched in specifically antigen-stimulated cells. These cells were obtained as educated T-cells by the method described by Mitchell and Miller (1968). The antigen used was SRBC and these cells are considered to be helper T-cells (Hunter *et al.*, 1972).

The educated T-cells did not show very significant changes in their EPM upon treatment with Con A under capping conditions (figure 5). Furthermore, they were unable to undergo any appreciable degree of ligand-induced redistribution (8%) when incubated with low concentration of FITC-Con A (table 2). In this respect they differ from the leukaemic cells or sub-population B in the thymus which show capping at low and moderate concentrations of Con A respectively but the high concentrations of lectin restrict the lateral mobility of their receptors.

Discussion

The foregoing data demonstrated that normal and developing cells differ in the nature and lateral mobility of their receptors to Con A. These alterations are reflected in their characteristic electrokinetic profiles as also in microscopically discernible receptor redistribution patterns. Whereas normal lymphocytes displayed two behaviourally distinct types of receptor sites (Sainis *et al.*, 1979a, b; Sainis and Phondke, 1980), the highly proliferating leukaemic cells of the same strain of mice displayed a single set of receptor sites for Con A. The lateral mobility of these sites was restricted by high concentrations of Con A (Sainis *et al.*, 1982).

Cells undergoing normal growth and differentiation were marked for their cellular heterogeneity on account of Con A-induced receptor redistribution. This cellular heterogeneity observed in the adult and developing thymus cells was based on the presence of two major sub-populations. One of them shows receptor characteristics similar to those in adult peripheral T-lymphocytes, while the other largely resembles proliferating leukaemic cells. Several workers have earlier shown mouse thymocytes to be heterogenous with respect to cortisone resistance (Papiernik *et al.*, 1977) PNA-agglutinability (Reisner *et al.*, 1976), mitogen responsiveness, location (Shortman *et al.*, 1977) and surface markers (Boyse *et al.*, 1968; Leckband and Boyse, 1971). Our present investigation has brought to light yet another parameter for cellular heterogeneity *viz.* Con A—induced receptor redistribution and resultant changes in EPM.

The peripheral T-lymphocytes are considered to be at the end of the differentiation pathway. In adult unimmunised mice they will represent a differentiated non-proliferating population of cells. The leukaemic cells on the other hand, are highly proliferating (Joshi *et al.*, 1983) and are not terminally differentiated. In the adult thymus only a small fraction of cells have been observed to undergo active proliferation (D. S. Joshi, M. Rajadhyaksha and G. P. Phondke, unpublished observations). The cells of sub-population *A* within the adult thymus which undergo Con A—induced redistribution at low concentrations of the lectin have receptor sites which share the properties of differentiated, non-proliferating peripheral T-cells. However, they differ from the latter in that the thymic cells were inhibited from Con A induced redistribution by high concentrations of the lectin. The cells of sub-population *B* in the adult thymus show receptor characteristics similar to those of the highly proliferating but undifferentited leukaemic cells. Here again, the main difference was only in the concentration at which the leukaemic cells and those of sub-population *B* showed capping (Sainis *et al*, 1982, figure 3). On the basis of the profiles of Con A-induced receptor-redistribution and the nature of the receptors, it may thus be possible to ascribe differentiational status to various sub-populations of lymphoid cells. In the present system, sub-population *A* in the thymus would then be considered more differentiated than sub-population *B*, but less differentiated than the peripheral T-cells.

Since the hydrocortisone-resistant thymocytes also retain the property of restricted mobility of surface receptors bound to high concentrations of Con A, they may also be considered less differentiated than the peripherl T-lymphocytes.

In the thymuses of growing mice there is a considerable amount of cellular traffic. There is also considerable higher degree of cell proliferation and differentiated cells continue to migrate out to peripheral lymphoid organs. This leads to cellular heterogeneity which can arise from the two sub-populations described in adult thymocytes. In the developing thymus the proportions of the two sub-populations at different levels of differentiation will also vary. This is borne out by pronounced bimodal EPM profiles following treatment with Con A. The proposition that differentiational status is related to the nature and lateral mobility of the receptors for Con A, derives further support from the observation that lymphocytes differentiated in response to a specific antigen show restriction of the mobility of receptors to polyclonal ligands like Con A even at low concentrations (figure 5; table 2).

Our data thus indicate that electrokinetic measurements on the interaction of Con A could be used as a structural probe for alterations in lymphoid cell surfaces following normal growth and differentiation and abnormal proliferation or specific maturation.

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Towards a subunit influenza vaccine prepared by affinity chromatography on immobilized lectin

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Abstract. Disintegration of influenza virions with 0.2% w/v sodium deoxycholate releases haemagglutinin and neuraminidase, which in the presence of detergent are both adsorbed to the lectin from *Crotalaria juncea*, coupled to Sepharose 2B.

The binding is mediated through galactosyl residues on haemagglutinin and neuraminidase, which are completely displaced in 0.2 *M* lactose and co-elute in a narrow zone.

The immunogenicity of haemagglutinin and neuraminidase in mice is markedly increased after adsorption onto lipid particles constituting "intralipid" (Kabi Vitrum, Stocholm, Sweden).

Keywords. Influenza subunit vaccine; haemagglutinin; neuraminidase; lectin; *Crotalaria juncea*; affinity chromatography; adjuvant.

Introduction

There are three principal types of influenza vaccine in present use: whole virus vaccine, split vaccine, and subunit vaccine. The advantages most often cited in favour of a subunit vaccine containing only haemagglutinin (HA) and neuraminidase (NA) are the following ones: 1. Lower toxicity leads to fewer local reactions at the site of injection. 2. Since irrelevant material has been eliminated, higher doses of the two relevant immunogens HA and NA can be administered. 3. Inoculation of ostensibly harmless but potentially active nucleic acid is avoided. Although local and systemic reactions against whole virus vaccines purified by zonal centrifugation are rarely serious in adults, clinical symptoms like swelling, pain, and moderate pyrexia are common in children and unprimed recipients (Tyrrell and Smith, 1979). These reactions can be reduced but hardly eliminated in "split" vaccines prepared by disintegration of purified virions with ether or detergents, which are afterwards removed. Split vaccines contain essentially the same elements as whole virus vaccines, and in the same proportions.

There is now ample evidence that the viral constituents of importance in eliciting protective humoral antibody are the surface glycoproteins HA and NA

Abbreviations used: HA, Haemagglutinin; NA, neuraminidase; Dol, Dolichol; Asn, asparagine; Ser, serine; Man, mannose; Glc, glucose; Glc N-Ac, glucose N-acetylamine; Thr, threonine; M_r, molecular weight; TNBP, tri-*n*-butylphosphate; CTAB, Cetyl trimethyl ammonium bromide; SDOC, sodium deoxycholate.

hild, 1979), and several experimental and few commercial vaccines based on
se purified immunogens have been tried.

Unfortunately, a weak and wavering immune response has been a frequent
oblem with the subunit vaccines. Reports are conflicting with respect to the level
well as the duration of the immune response to purified HA and NA subunits
m different virus strains both in man and experimental animals. Taken
gether, available evidence strongly suggests that the prospects for subunit in-
enza vaccines are bleak unless some way to potentiate and stabilise the immu-
genicity can be found. Several types of adjuvants have been suggested and a few
ve been actually tried in man, but to our knowledge none has yet given results
at are consistent enough to firmly establish subunit vaccines as being clinically
eferable to inactivated or live, attenuated whole virus vaccines.

We have recently had some success with a lipid based adjuvant, "intralipid", ori-
nally designed and introduced by Vitrum, as a nutritional aid for intravenous
e. As an adjuvant it may be more readily acceptable, since it is cleared for human
e although for an entirely different purpose.

Influenza virus HA is synthesized in the infected cell as a single polypeptide
ain to which carbohydrate side chains are added at asparagine residues (Klenk,
980) by the precursor-phosphoryl dolichol pathway (Struck and Lennarz, 1980).
ost of the efforts to elucidate the biosynthesis, structure, and function of the two
omponents have been directed towards HA, but the general pattern appears to be
milar in NA (Skehel *et al.*, 1980). In HA, assembly of the peptide chain starts in
e rough endoplasmatic reticulum. Attachment of carbohydrate begins by the
ddition of a preformed Dol-P-P-(GlcNAc)$_2$-Man$_9$-Glc$_3$ unit exclusively to aspa-
gine in the sequence Asn-X-(Ser or Thr). A series of events then follows in
hich the chronology and cellular locations are not known in detail. After initial
ttachment of the lipid-carbohydrate precursor, the glucosyl and a variable num-
er of mannosyl residues are removed. During passage through the Golgi mem-
ranes the majority of the trimmed carbohydrate chains are processed further by
equential addition of GlcNAc and then galactose. Sometimes one fucosyl residue
put on to one of the Asn-linked GlcNAc residues. Sialic acid is never present in
he finished molecule, which is a trimer of M_r 225,000 consisting of identical sub-
nits held together by noncovalent forces (Waterfield *et al.*, 1979).

There are two main types of carbohydrate chains. Complex chains contain all
he four sugars GlcNAc, mannose, fucose, and terminal galactose, whereas in high
nannose chains there are only GlcNAc and a variable number of terminal
nannosyl residues. The number of carbohydrate chains seems related to the num-
er of glycosylation sites in the polypeptide chains of a particular virus strain
Klenk, 1980).

The location of glycosylation sites and number of carbohydrate chains in NA
ave not been as intensively studied as in HA. Total weight of carbohydrate in NA
s about 10% compared to 20% in HA (Skehel *et al.*, 1980). The finished NA mole-
ule is a tetramer of four coplanar units with a total M_r of 270,000 (Skehel *et al.*,
1980). Therefore, the terminal sugars galactose and mannose as well as the
ranched GlcNAc-linked fucose are potentially exploitable for lectin affinity chro-
natography both of HA and NA.

Experimental

Backed by facilities at the Separation Center of the Biomedical Center for preparation of large amounts of lectin, and with experience in using immobilised lectins for purification of blood group substances and other glycoproteins, we decided about seven years ago to look more closely into the possibility of using lectins to purify intact virus and glycosylated viral subunits on a large scale.

This approach to the preparation of vaccines had already been suggested by Hayman *et al.* (1973) who described the isolation of HA and NA from the strains Ao/Bel/42, X-31, and Rostock Fowl Plague by affinity chromatography on *Lens culinaris* lectin coupled to Sepharose 4B with cyanogen bromide.

Aiming for a large scale method that would have to be scientifically sound, technically feasible, and economically acceptable at the same time, we had to start practically from scratch with all the three parts of the project: a) Disruption of virions; b) Lectin affinity chromatography; and c) Immune potentiation.

Disruption of virions

A multitude of procedures have been described for releasing HA and NA from the outer lipid layer of the influenza virion. They include the use of ethyl ether, either alone (Cromwell *et al.*, 1969), or in combination with detergents (Davenport *et al.*, 1964); tri-*n*-butyl phosphate (TNBP) (Neurath *et al.*, 1970, 1971), as well as cationic, neutral, and anionic detergents. The purification steps following general disruption with a non-selective detergent have involved density gradient centrifugation (Brady and Furminger, 1976), electrophoresis (Laver *et al.*, 1976), both in combination (Laver and Webster, 1976a, b), alcohol precipitation and ultracentrifugation (Janout and Uvizl, 1977), or centrifugation after selective solubilisation with the cationic detergent cetyl trimethyl ammonium bromide (CTAB) (Bachmayer *et al.*, 1976).

The method involves treatment of freshly harvested allantoic fluid with 0.2% w/v of the anionic detergent sodium deoxycholate (SDOC) under gentle stirring for 12 h at room temperature and pH 8.0. This concentration of SDOC is low compared to what other workers have used (Ersson, 1977), but we found it to be the upper limit for preservation of both HA and NA activity in H1N1 strains, which appeared particularly sensitive to SDOC. We observed no turbidity or gel formation with 0.02 M phosphate as present in phosphate buffered saline at this concentration of SDOC. The effect of pH on the efficiency of HA and NA release in 0.2% SDOC varied between virus strains. At pH levels down to 7.4 the H3N2 strains like A/Victoria/3/75 and A/England/42/72 were effectively disrupted, whereas only partial release was obtained from the H1N1 A/USSR strains until pH was increased to 8.0.

Results

Lectin affinity chromatography

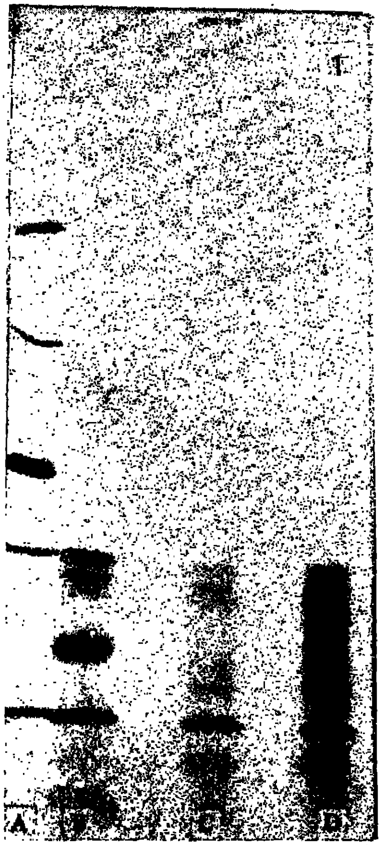
Potentially useful lectins for the isolation of HA and NA subunits are those with affinity for mannose, galactose, or fucose. In addition to proper specificity, several other qualities are essential in lectin-based adsorbents to be used for preparation of injectable material. The lectin must be non-toxic, and continuous background leakage of lectin should either lie below the level of immunogenicity, or else the lectin leaking off during desorption should be easily removable. Further, the capacity should be high enough to allow a reasonable volume ratio of adsorbent to vaccine and must be retained through several cycles of adsorption and desorption. Finally, complete desorption with cheaply available carbohydrate should be possible. We found that different lectins of similar sugar specificity could vary considerably in their ability to adsorb and release virions and subunits. Experience with some lectins is summarised in table 1.

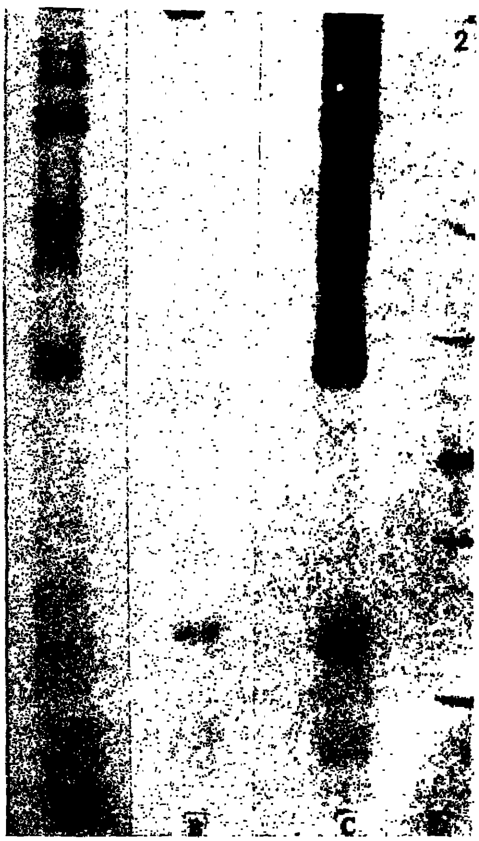
Table 1. Affinity chromatography of virus and subunits on different immobilised lectins.

Lectin	Interacting sugar on HA and NA	Virus material	
		Intact virions	HA+NA
Concanavalin A	Mannose	Strong binding, incomplete release	Binding erratic, strain dependent
Lens culinaris	"	Strong binding, incomplete release	Progressive decrease in binding of NA
Vicia ervilia	"	Good capacity in repeated use	Binding too weak
Crotalaria juncea	Galactose	Strong binding, some progressive decrease in binding of HA and NA	Excellent capacity in repeated use
Lotus tetragonolobus	Fucose	Binding too weak	Binding too weak

For affinity chromatography of HA and NA subunits, the most useful lectin we have tried so far is that from *Crotalaria juncea* (sunn hemp), which has been extensively purified and characterised by Ersson (1977) at the Separation Center. A welcome property of this lectin is its high affinity for lactose, which inhibits haemagglutination about eight times more efficiently than does galactose. This means that HA and NA when adsorbed through their terminal galactosyl residues are very efficiently desorbed by 0.2 M lactose in phosphate buffered saline and eluted in a narrow zone at high concentration.

Pilot experiments showed that the presence of 0.2% SDOC had little effect on the carbohydrate-binding capacity of lectin-Sepharose. Figure 1 shows the pattern obtained after polyacrylamide gradient electrophoresis of the starting material before (lane B) and after (lane C) treatment with SDOC. Lane D represents unadsorbed material passing through the column, but with no residual HA or NA activity. Lanes B and C show that even in the absence of detergent the bulk of allantoic fluid material appears below the lactic dehydrogenase marker of M_r 140,000. Lanes C and D are very similar, suggesting that the adsorbent binds only a small fraction of the protein present in the starting sample. In figure 2 desorbed material from infected (lanes A and C) and non-infected (lane B) allantoic fluid is compared. In the absence of SDOC due to thorough flushing before desorption,





Figures 1 and 2. 1. Polyacrylamide gradient electrophoresis of infected allantoic fluid, Coomassie stain.

Lane A: M_r markers (Pharmacia Fine Chemicals), top to bottom, M_r in thousands: thyroglobulin 669, ferritin 440, catalase 232, lactate dehydrogenase 140, albumin 67.

Lane B: Crude allantoic fluid before SDOC treatment.

Lane C: Crude allantoic fluid after SDOC treatment, as applied to lectin column.

Lane D: Material not adsorbed on lectin column, devoid of HA and NA activity.

2. Polyacrylamide gradient electrophoresis of material desorbed after passage of infected and non-infected allantoic fluid.

Lane A: Material desorbed with lactose after passage of infected allantoic fluid, PAS-Schiff stain.

Lane B: Material desorbed with lactose after passage of non-infected allantoic fluid, Coomassive stain.

Lane C: Same material as in lane A, but stained with Coomassie.

Lane D: Molecular weight markers (see figure 1, lane A).

most bands stained for proteins in lane C lie above the ferritin marker of M_r 440,000. Almost all appear to be glycoproteins as demonstrated by an aliquot run stained with PAS-Schiff in lane A. The presence of several bands is expected, since it has been generally found that oligomeric aggregates of HA and NA tend to form by hydrophobic association after the removal of detergent.

Lane B is almost empty, sharing a conspicuous band with lane C at around M_r 90,000. The tentative conclusion is, that most of the material released from the lectin-Sepharose by lactose, after passage of infected allantoic fluid treated with SDOC, is indeed of viral origin.

Lectin toxicity and stability of immobilised lectin

Two conditions essential to the acceptance of lectin chromatography in providing injectable preparations for human use are a lack of lectin toxicity and a lectin content per dose that does not elicit unacceptably high levels of anti-lectin antibody.

The *Crotalaria juncea* lectin is not toxic in mice. No LD-50 could be established even with doses corresponding to 10 mg/kg body weight.

In order to determine the ratio to be expected between viral subunits and background lectin, leakage of lectin labelled with ^{125}I was measured under different conditions and during various stages of the chromatographic procedure. There is some release of lectin when the crude mixture of allantoic fluid and detergent passes through. Apart from a slight progressive decrease in capacity to be expected from this, our main concern was of course the amount of lectin appearing with the subunits in the desorption zone. Preliminary results indicate that a vaccine dose containing 15 μg HA may contain about 0.07 μg lectin, although optimum conditions for production runs have not yet been established.

Immunisation experiments

We realised at an early stage of our efforts that an efficient adjuvant suitable for human use was likely to be required for a practical subunit vaccine to be justified. During discussions about the contribution of hydrophobic bonding in chromatographic systems the hydrophobic tails of viral HA and NA was a recurrent topic. Our attention was drawn to a remarkably stable soybean oil emulsion introduced into the market as an intravenous nutrient called "intralipid" and cleared for human use. Although stabilised with lecithin, the particles differed from liposomes in being essentially homogeneous rather than consisting of a lipid bilayer surrounding a water phase. Since the soybean oil droplets forming an oil-in-water emulsion were about equal to influenza virions in size and could be expected to have a hydrophobic surface, we decided to make a mixture of intralipid and viral subunits, inject it into mice, and look at the immune response.

A similar approach was taken by Almeida *et al.* (1975) who describe the preparation of "virosomes" consisting of influenza viral HA and NA adsorbed to the surface of unilamellar liposomes presumably by hydrophobic bonding and looking remarkably like viral particles in the electron microscope. However, apparently no later data have been published on the practical use of these virosomes.

Preliminary results with intralipid as an adjuvant in immunisation of mice with influenza HA and NA are very encouraging indeed. Parameters have not yet been optimised, but some figures are given in table 2.

The future

The main challenges are to secure a steady supply of lectin, to establish optimum chromatographic conditions, and to optimise and stabilise the adjuvant effect. Also, there is no guarantee that intralipid is as immunologically harmless in humans as it appears to be in mice after conjugation with viral immunogens. Finally,

Table 2. Haemagglutination inhibition titrations of sera from mice immunized with whole virus or HA+NA subunits with and without addition of "intralipid".

Material injected	μg protein per dose (0, 2 ml) before dilution	Antigenic extinction limit[a]	
		Three weeks after first injection	One week[b] after second injection
Current divalent whole virus vaccine without intralipid (A/Vic/375+B/HK/8/73B)	ND[c]	1:150	1:1400
Whole virus vaccine+intralipid	ND	1:18600	1:49000
A/Vic HA+NA subunits without intralipid	130	1:390	ND
HA+NA subunits in 2% intralipid	130	1:25700	1:178000

[a] The dilution of injected material causing detectable antibody formation in 50% of injected animals

[b] Second injection was given after first bleeding

[c] Not determined

the crucial question of how efficiently the vaccine protects against infection in man remains to be answered.

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The role of eucaryotic cell surface glycoconjugates in DNA replication

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Abstract. Multiple forms of ricin have been isolated from castor bean seeds. Two forms, ricin-1 and ricin-2, differ in their isoelectric pI values and toxicity towards IMR-32 cells. Inhibition of IMR-32 DNA polymerase α_2 is more pronounced with ricin-1 (65%) than with ricin-2 (10%). Ricin B chain (pI – 5.2) isolated from ricin-1 binds to IMR-32 cell surfaces as well as inhibits DNA polymerase α_2 activity when studied *in vitro*. The presence of galβ-linked glycoconjugates near the active site of IMR-32 DNA polymerase α_2 has been proposed. Replication modulators which bind to the glycose portion of the enzymes involved in the replication system may need a mandatory binding to cell surface glycoconjugates for their activity.

Keywords. Cell surface; DNA biosynthesis; DNA polymerase α; glycoconjugates; IMR-32 cells; ricin b; neuroblastoma cells.

Introduction

The complex biochemical steps that lead to the control of chromosomal DNA replication during differentiation of eucaryotic cells are not yet understood. To clarify the mechanism of expression of various properties of differentiated neurons, many studies have been performed with mouse neuroblastoma cells (Augusti-Tocco and Sato, 1969; Amano *et al.*, 1972; Prasad and Sinha, 1978) or neuroblastoma *x* glioma hybrid cells (Nelson *et al.*, 1976; McGee *et al.*, 1978). These cells are induced to produce neurites in the absence of serum (Amano *et al.*, 1972; Basu *et al.*, 1976a; Prasad and Sinha, 1978 or in the presence of inhibitors of 3′,5′-cyclic AMP (cAMP) phosphodiesterase (Basu *et al.*, 1976a; Moskal, 1977; Prasad and Sinha, 1978) or DNA biosynthesis (Schubert and Jacob, 1970; Moskal, 1977; Bhattacharya and Basu, 1978). Many factors appear to be involved in this process. During differentiation of neuronal (Basu *et al.*, 1976a; Prasad and Sinha, 1978) or non-neuronal cells (*e.g.* CHO cells, Hsie and Puck, 1971), cAMP seems to play an important but as yet uncharacterized role (Prasad and Sinha, 1978). Phosphorylation (Prasad and Sinha, 1978) and acetylation (Levy *et al.*, 1975; Ruben *et al.*, 1976; Marks *et al.*, 1978; Vidali *et al.*, 1978) of specific histones have been suggested as possible mechanisms involved in the arreast of cell division during differentiation of Friend virus-transformed murine erythroleukemia cells induced by dimethylsulfoxide or hexamethylenebisacetamide.

Alternatively, modification of the cell surface glycoconjugates may serve to increase or decrease the transport of serum nutrients or growth factors into the cell, leading eventually to re-initiation or "shutting off" of the cell cycle, as suggested by Holly (1972). Perhaps proteins synthesized inside the cytoplasm (endogenous) or coming from outside the cell (exogenus) through a specific transport system present on the cell surface may play direct roles in DNA replication.

CHaracterization of galactose-containing glycoconjugates on neuroblastoma cell surfaces

Knowledge of the chemistry and biosynthesis of glycoconjugates present on neuroblastoma cell surfaces is still in its infancy. Recent studies from different laboratories (Rosenfeldor *et al.*, 1977; Hakomori *et al.*, 1980; Basu *et al.*, 1980) have suggested that the distribution of glycoconjugates on tumor cell surfaces differs from that of normal cells. We thought in the early 1970's that the evaluation of such data would be facilitated by a systematic study of the phylogenetic distribution of the enzymes involved in the synthesis of cell-surface glycoconjugates. During the past ten years we have collected information about the relative activities of glycosyltransferases present in neuroblastoma cells of primate (human IMR-32) and nonprimate (mouse N-18) origins (Prasad and Hsie, 1971; Moskal *et al.*, 1974; Basu *et al.*, 1976a; Presper *et al.*, 1978). We have established the enzymatic pathway for the biosynthesis of the fucose-free B-active pentaglycosylceramide nLcOse$_5$ Cer (Galα1-3Galβ1-4GlcNacβ1-3Galβ1-4Glc-Cer; figure 1) in both mouse N-18 and human IMR-32 neuroblastoma cells. In each case, synthesis takes place by stepwise elongation of the oligosaccharide chain by a specific glycosyltransferase (figure 2). Expression of an (α1-2) fucosyltransferase required for the synthesis of terminal fucose-containing blood group-related glycosphingolipids (Basu *et al.*, 1975) appears to be confined to cultured cells of primate origin (Presper *et al.*, 1978).

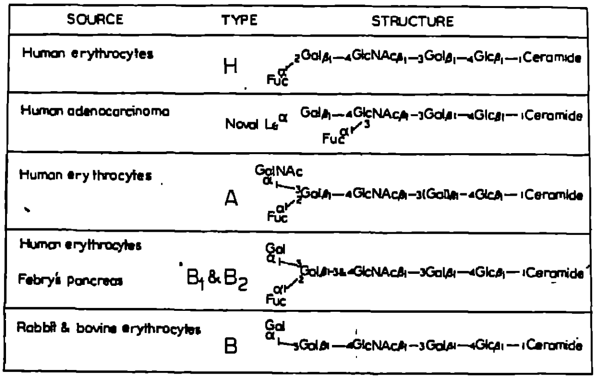
SOURCE
TYPE
STRUCTURE
Human erythrocytes
H
Galβ1—4GlcNAcβ1—3Galβ1—4Glcβ1—1Ceramide
Fuc
Human adenocarcinoma
Noval Le^α
Galβ1—4GlcNAcβ1—3Galβ1—4Glcβ1—1Ceramide
Fuc
Human erythrocytes
A
GalNAc
Galβ1—4GlcNAcβ1—3(Gal)β1—4Glcβ1—1Ceramide
Fuc
Human erythrocytes
Febry's pancreas
B1 & B2
Gal
Galβ1-3&4GlcNAcβ1—3Galβ1—4Glcβ1—1Ceramide
Fuc
Rabbit & bovine erythrocytes
B
Gal
Galβ1—4GlcNAcβ1—3Galβ1—4Glcβ1—1Ceramide

Figure 1. Structures of blood group active glycosphingolipids.

Using specific lectins from *Bandeiraea simplicifolia* and *Ricinus communis*, we have established that these B-specific glycosphingolipids are exposed on the cell surfaces of both mouse and human neuroblastoma cells (Prasad and Hsie, 1971; Basu *et al.*, 1976a, b). Recently, using [^{125}I]-ricin, we have proved that both types of cells contain receptor sites which bind the ricin *B* subunit. On the basis of inhibition studies with methyl-β-D-galactopyranoside (Bhattacharya *et al.*, 1980), these receptor sites appear to be glycoconjugates containing terminal β-linked galactose. It is important to note that these cells contain high activities of UDP-galactose: LcOse$_3$Cer (β1-4) galactosyltransferase (Basu and Basu, 1972; Presper *et al.*,

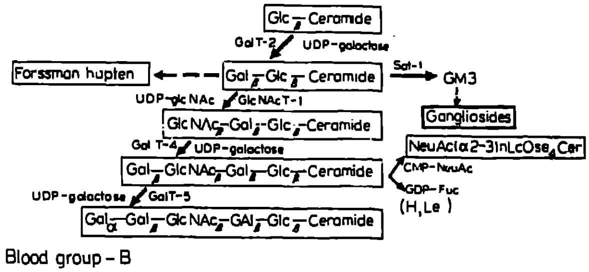

Figure 2. Proposed pathway for biosynthesis of blood group related glycosphingolipids.

1978), which catalyzes the synthesis of $nLcOse_4$ Cer (Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer; figure 2), an important intermediate of many blood group-active glycosphingolipids. Recently, Sundsmo and Hakomori (1976) have identified this glycosphingolipid as a potential tumor-associated antigen of polyoma-transformed NIL (NIL_{py}) cell surfaces.

Although specific glycosphingolipids have been identified on the cell surfaces of various chemically and virally transformed cells, their biological roles are not yet understood. Treatment of mouse neuroblastoma cells with the differentiating agent dibutyryl cyclin AMP (1 mM) reduces the binding of [^{125}I]-labelled *Bandeiraea simplicifolia* lectin (which recognizecs terminal α-linked galactose-containing glycoconjugates) to 30% of control values, suggesting a relationship between differentiation and cell surface composition. This is further supported by the findings of Nicolson *et al.* (1975), who demonstrated that an SV40-transformed 3 *T*3 cell line was more sensitive to the toxic effects of ricin than untransformed 3 *T*3 cells.

Lectin-induced inhibition of DNA synthesis

Ricin, a highly toxic glycoprotein isolated from castor beans (*Ricinus communis*), is a strong inhibitor of protein biosynthesis (Lin *et al.*, 1970; Olsnes and Pihl, 1973; Hedblom *et al.*, 1978). It also inhibits [^{3}H]-thymidine incorporation into chromatin DNA of tumor cells (Lin *et al.*, 1971). Previous studies on growth control and toxicity of ricin in cultured neuronal cells (Gonatas *et al.*, 1975) suggest receptor-mediated endocytosis of intact ricin (M_r, 64,000). Inhibition of protein biosynthesis in cell-free systems in the presence of the ricin *A* subunit has been reported by several laboratories, whereas the *B* subunit was generally assumed to have no function other than binding to the cell surface.

Early investigations in our laboratory on the effects of various lectins and toxins on the incorporation of [^{3}H]-thymidine into the DNA of human IMR-32 (Bhattacharya *et al.*, 1979) and mouse N-18 (unpublished) neuroblastoma clones revealed that total cellular DNA synthesis was reduced by concanavalin A and ricin. The failure of added serum to reverse the ricin-mediated inhibition of DNA synthesis suggested two possible mechanisms for its action: either ricin, by combining with the cell surface (Nicolson *et al.*, 1975), influenced the ability of the cell to respond to agents that initiate DNA synthesis, or it entered the cell (Gonatas, 1980) and directly inactivated one or more components of the DNA replication complex. The latter hypothesis was supported by our demonstration that ricin specifically inhibited human neuroblastoma DNA polymerase α activity *in vitro*, while DNA polymerase β activity was unaffected (Bhattacharya *et al.*, 1979).

The existence of multiple forms of ricin has been reported (Olsnes and Pihl, 1973; Hedblom *et al.*, 1978). We have observed that differential toxicities toward cultured neuroblastoma IMR-32 cells among these multiple forms are due to differences in their *B* subunits. Recently, we have isolated different *B* chains with characteristics iso-electric points, and we are now studying their effects on the activities of multiple forms of DNA polymerase α.

Multiple forms of DNA polymerase α from human neuroblastoma IMR-32 cells

Multiple forms of DNA polymerase α have been identified in animal tissues (Momparler *et al.*, 1973; Hesslewood *et al.*, 1978; Yoshida *et al.*, 1974), solid tumors (Matsukage *et al.*, 1974; Chen *et al.*, 1979), and cultured tumor cells (Tanabe and Takahashi, 1976; Bieri-Bonniot and Schuerch, 1978; Ono *et al.*, 1979). Whether these forms have different primary structures, or whether they arise through modification of a common parental form is not yet known. Wilson and his co-workers (Chen *et al.*, 1979) recently purified to homogeneity from the soluble protein fraction of mouse myeloma two species of DNA polymerase α which consists of non-identical subunits with apparent molecular weights of 47,000 and 54,000. Tryptic peptide mapping of these two polymerases failed to show any sequence homology between the two subunits, although the intact enzymes share similar subunit molecular weights, native molecular weights, and catalytic properties. In contrast, Johnston and co-workers (Hesslewood *et al.*, 1978) showed that mild urea treatment of one form of calf thymus DNA polymerase α (*A* form, 8 S) generated a second form of the enzyme (*C* form, 7.3 S). They concluded that the *C* form was a single polypeptide and that the *A* enzyme contained an additional subunit of molecular weight 50,000–70,000. Whether the urea treatment dissociated a true subunit or a pre-existing proteolytic fragment is unclear.

Recently, we have reported (Bhattacharya *et al.*, 1980, 1981, 1982) the isolation and characterization of three forms (α_1, α_2, α_3) of DNA polymerase α from IMR-32 human neuroblastoma cells. These forms display differential template specificities and heat sensitivities. The α_1 form very efficiently utilizes poly $(dA)\cdot(dT)_{12\text{-}18}$ poly$(dA\text{-}dT)$, and activated calf thymus DNA as template-primers, while no activity could be detected using poly $(rA)\cdot(dT)_{12\text{-}18}$. DNA polymerase α_2 appears to utilize the poly$(rA)\cdot(dT)_{12\text{-}18}$ template-primer to a small but significant extent in addition to incorporating high levels of [^{3}H]-labelled deoxynucleotides into those templates preferred by the α_1 form. DNA polymerase α_3 exhibits maximal activity with poly$(dA)\cdot(dT)_{12\text{-}18}$ and poly$(dA\text{-}dT)$; it also uses both activated calf thymus DNA and poly$(rA)\cdot(dT)_{12\text{-}18}$, but with considerably lower efficiency than forms α_1 and α_2.

These three forms are differentially inhibited by the ricin *B* subunit and by a nonhistone chromatin protein isolated from rat liver (Bhattacharya *et al.*, 1978, 1981). These findings suggest that the multiple forms may have different physiological functions in the replication of DNA in IMR-32 cells. This conclusion is supported by the observation of changes in the levels of two forms of DNA polymerase α (*P*-I and *P*-II) during the HeLa cell cycle made by Ono *et al.* (1979) *P*-I activity increases between the G_1 and S phases, reaches a maximum at mid-S phase, and then declines rapidly. *P*-II activity is fairly constant throughout the cycle. The decrease in *P*-I activity may be due either to the appearance of an inhibitor or to decreased synthesis of the enzyme.

Conclusion

The overall objective of our research is to understand the complex biochemical events that lead to the initiation and termination of DNA replication in neuroblastoma cells during differentiation. Perhaps proteins synthesized in the cytoplasm (endogenous) or coming from outside the cell (exogenous) after binding on the cell surface may play direct roles in the control of DNA synthesis. Our recent studies on the inhibition of DNA polymerase α by an acidic nonhistone chromatin protein (NHCP) (Bhattacharya *et al.*, 1978, 1981) and by ricin will serve as model systems for endogenous and exogenous effector proteins, respectively. The acidic protein (NHCP; M_r, 23,000) isolated from a mixture of rat liver chromatin proteins inhibits IMR-32 DNA Polymerases α_2 and α_3 by 85% and 83%, respectively. The nature of inhibition by this endogenous protein is a subject of our current investigation. Ricin is an ideal model for an exogenous protein which controls DNA synthesis. It binds specifically with terminal β-linked galactose-containing glycoconjugates, which are found in a wide variety of cell surfaces. Also, it specifically inhibits DNA polymerase α (Bhattacharya and Basu, 1982) isolated from human neuroblastoma IMR-32 cells. A similar reduction of neuroblastoma DNA polymerase α activity was observed (Bhattacharya and Basu, 1978) by treatment of mouse *N*-18 neuroblastoma cells with the differentiating agent 5-bromodeoxyuridine (BrdUrd). The steps that result in the reduction of chromosomal DNA replication in the presence of BrdUrd are not yet known. On the basis of our inhibition studies with ricin we propose a model for the control of DNA replication by an exogenous protein (figure 3). It appears that cell-surface glycoconjugates may play an important role in recognition of such exogenous proteins.

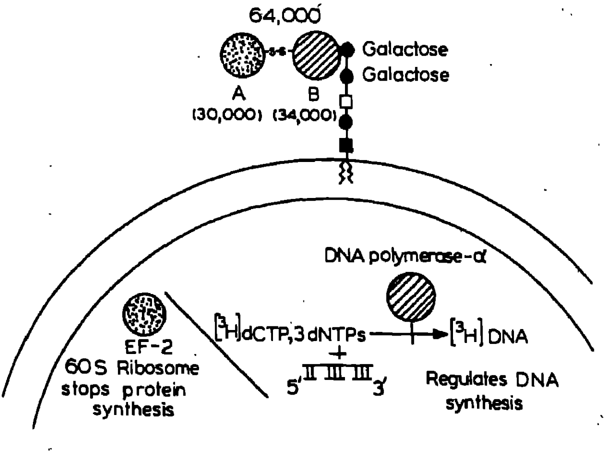

Figure 3. Proposed mechanism of ricin toxin action.

The expression of a specific cell-surface glycosphingolipid or glycoprotein during differentiation or transformation could therefore be a crucial step (figure 4) in the control of DNA replication by an exogenous protein.

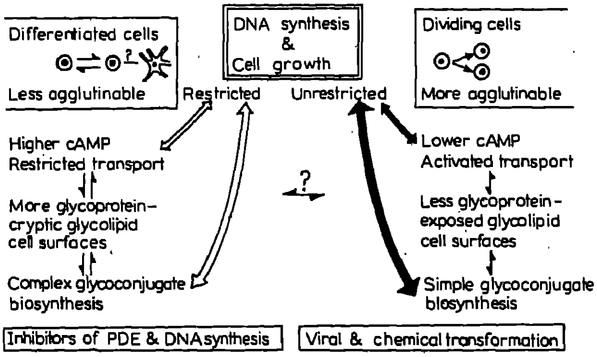
Differentiated cells
Less agglutinable
DNA synthesis & Cell growth
Dividing cells
More agglutinable
Restricted
Unrestricted
Higher cAMP
Restricted transport
More glycoprotein-cryptic glycolipid cell surfaces
Complex glycoconjugate biosynthesis
?
Lower cAMP
Activated transport
Less glycoprotein-exposed glycolipid cell surfaces
Simple glycoconjugate biosynthesis
Inhibitors of PDE & DNA synthesis
Viral & chemical transformation

Figure 4. Interrelationship between cell growth and cell surfaces.

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